Evaluation of Pollen-Mediated Gene Flow and Seed-Associated Microbial Communities in Northern Wild Rice (*Zizania palustris* L.)

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Abstract

As the official state grain of Minnesota, northern wild rice (NWR; *Zizania palustris* L.) represents an economically important specialty crop with a rich cultural heritage. Native to the Great Lakes region of North America, domestication of the plant began in the 1950s and today, the vast majority of cultivated NWR is produced in Minnesota and California (Zepp, Harwood, & Somwaru, 1996).

Like maize, NWR is an open-pollinated crop, making pollen difficult to control. NWR flower morphology promotes out-crossing, thus necessitating an understanding of pollen travel. By understanding pollen travel, the breeding program is ultimately able to understand spatial limitations while developing inbred lines and maintaining varietal purity. Here, the characterization of a recessive white male floret (WMF) population in contrast to the dominant, purple male floret (PMF) color of cultivated NWR along with estimates of pollen-mediated gene flow in a cultivated paddy setting is presented. Studies revealed that the primary amount of pollen-mediated gene flow occurred within the first 7 m from the PMF donor source with no gene flow detected past 63 m. This study demonstrates that the WMF trait is an excellent candidate for use in pollen-mediated gene flow studies in NWR.

As a wild crop relative of *Oryza sativa*, the Zinaniiae subtribe within Oryzaea has seed that is desiccation-sensitive, which is unique among grain crops. Additionally, seed must be stored submerged at ~3 °C for a period of three to four months to help break dormancy, after which time seed still may not germinate. Commercial NWR seed germination rates range from 15% to 95%, with only approximately 60% of germinated seeds going on to establish plants. In research settings, the wet storage environment is

optimal for microbial growth, but the effect microbial growth on NWR germination/dormancy is currently unknown. While several fungal diseases have been noted to plague plants of cultivated NWR, diagnoses of stored seed diseases are not widely known in the research community. In this study, we isolated and sequenced microbes found in 27 NWR seed stocks collected over a five year period from three locations and four genotypes (three released NWR varieties and one elite breeding line). Results revealed that microbial communities were heavily dependent on seed viability and began shifting after one year of NWR seed storage. We also evaluated the efficacy of four antimicrobial treatments for the reduction of microbial growth in hydrated NWR seed storage. These treatments did not reduce or drastically change microbial growth or seed viability. Overall, this study introduces common microbial constituents found in the seed storage of an aquatic, cold-adapted, recalcitrant species and provides a foundation for future studies to evaluate the effect of microbial communities on NWR seed viability during hydrated storage.

Ultimately, these results suggest that the likelihood of pollen-mediated gene flow between cultivated NWR and natural stands remains low, reducing the perceived threat to growers, conservationists, or concerned communities. Additionally, antimicrobial seed treatments are not an effective mechanism of controlling microbial growth in NWR seed storage, but the identification of the core NWR microbiome will be useful for researchers going forward.

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Zizania palustris: A Species Overview

As the official state grain of Minnesota, northern wild rice (*Zizania palustris* L.) represents an economically important specialty crop with a rich cultural heritage. Native to the Great Lakes region of North America, northern wild rice (NWR) is one of four species belonging to the genus *Zizania*, which is a part of the Poaceae family. Poaceae is the most economically important plant family, as it includes staple foods like maize, rice, wheat, barley, and millet. Of the 43 tribes in Poaceae (Watson, 1992), the Oryzeae tribe is divided into two subtribes, separated by their flower morphologies—Oryzinae (perfect flowers) and Zizaniinae (imperfect flowers but still monoecious) (Guo & Ge, 2005; Hitchcock & Chase, 1951; Stebbins & Crampton, 1961). White rice belongs to the Oryzinae subtribe, while NWR belongs to Zinaniinae, but despite their separation into taxonomic subtribes, the genus *Zizania* is the closest genus to *Oryza* phylogenetically, with the addition of the genus *Leersia* (Kellogg, 2009). Previously, there was some contention on the placement of NWR into Oryzeae and further into Zizaniinae, however this placement was confirmed with the release of the NWR genome (Duvall, Peterson, Terrell, & Christensen, 1993; Ge, Li, Lu, Zhang, & Hong, 2002; Haas et al., 2021; Tang et al., 2010). NWR is considered a crop wild relative (CWR) of white rice (*Oryza sativa* L.) (Harlan, 1976; Khoury et al., 2013). The close taxonomic relationships and collinearity between white rice and *Zizania* species has allowed for their effective comparative mapping, especially as white rice has served as a model species for comparative mapping within Poaceae (Goff, 1999). The

initial genetic map of NWR was composed of previously mapped RFLP genetic markers from white rice, and nearly 85% of those common white rice RFLPs were collinear with NWR (Kennard, Phillips, Porter, & Grombacher, 1999). Because of their close synteny, genetic inferences and other genetic resources for NWR are gleaned from the *Oryza sativa* genome. *Zizania* species have also been utilized to contribute useful genes to white rice via genetic transformation, as *Zizania* species have comparatively higher cold-tolerance (Abedinia, Henry, Blakeney, & Lewin, 2000; Liu, Liu, & Li, 1999) and enhanced disease resistance (Chen, Long, Lin, Guo, & Liu, 2006; Liu et al., 1999). Breeding advancements, primarily via improved molecular approaches, have increased the efficiency of CWR use substantially (Ford-Lloyd et al., 2011; Tanksley & McCouch, 1997; Volk & Richards, 2011; Zamir, 2001).

Zizania species vary wildly in their geographic distribution, life cycle/life expectancies, and end uses. Geographically, the distribution of *Zizania* species is considered disjunct as one species' center of origin is separated by a considerable distance. In the case of *Zizania*, one species originated in Eastern Asia, while the remaining three species are native to North America. Historically, *Z. palustris* has been referred to with many different common names (wildrice, wild rice, wild-rice, water oat, American wild rice), however, Terrell (1997) summarizes a geography-specific naming scheme for *Zizania* species. *Zizania palustris* L. (northern wild rice), *Zizania aquatica* L. (southern wild rice), and *Zizania texana* Hitchc. (Texas wild rice) span much of the coastal areas of North America including the Great Lakes, the east coast of the United States, and Texas (Figure 1) (Porter, 2019). The fourth and final species, *Zizania latifolia* (Griesb) Turcz. ex

Stapf (Manchurian wild rice), is native to China. More specifically, *Z. palustris* is native to the great lakes region of North America. *Z. aquatica* is found along the eastern and southeastern coasts of the United States and Canada. *Z. texana* is native to a localized area of the San Marcos River in Texas, and is also an endangered species (Terrell, Peterson, Reveal, & Duvall, 1997). *Z. aquatica* and *Z. palustris* are both annuals, while *Z. texana* and *Z. latifolia* are perennial (Porter, 2019). Of the four species, *Z. palustris* is the largest seeded type, is diploid (2x=2n=30), and is the only species harvested for its grain (Oelke et al., 1982). *Z. latifolia* is not harvested for grain, but instead plant stems are infected with smut fungus (*Ustilago esculenta*), then eaten raw or cooked like a vegetable (Oelke & Schreiner, 2007; Terrell & Batra, 1982). Seeds produced by *Z. aquatica* and *Z. texana* are too small or thin to be effectively harvested for food (Oelke et al., 1982). It is NWR's role as a food source that has motivated agronomic and breeding research, and is the reason comparatively more research has been conducted on that species.

Unlike its relative white rice, NWR does not survive dryland cultivation like other crops. NWR completes its entire life cycle in slow-moving, shallow freshwater lakes and rivers (Figure 2). In natural settings during the spring months, seed along lake- and stream-beds begins to germinate when the water temperature reaches approximately 4 °C (Vennum, 1988) or when spring ice melt occurs (Atkins, Thomas, & Stewart, 1987) and grows towards the surface of the water. Once submerged leaves reach the surface, the plant begins putting out a waxy floating leaf, reaching Principal Phenological Stage (PPS) 1 (Duquette & Kimball, 2020). After reaching the floating leaf stage, tillering occurs (PPS 2) and the stem continues to elongate forcing leaves to grow aerially (PPS 3). After some

time, heading begins (PPS 5) and flowering takes place (PPS 6). Pollination occurs and the seed continues to develop (PPS 7) until it fully matures (PPS 8) and shatters off the plant back in to the stream- or lakebed (PPS 9). Cultivated NWR follows a similar timeline, however seed is stored imbibed in a more controlled environment over the winter. In late April/early May seed is spread or drilled into dry paddies or ponds that are flooded immediately after planting. Following the completion of flowering and pollination, the paddies are drained to create conditions suitable for harvesting machinery by the time the seed has matured. Prior to harvest, some seed has likely shattered into the paddy, either creating a volunteer population that will need to be controlled for the next season or aiding in improving the plant density of the next season's crop. After harvest, seed is stored submerged in nearby drainage ditches in permeable bags to overwinter. In research settings, harvested seed is stored submerged at 4 °C in a cooler (Grombacher, Porter, & Everett, 1997a; Oelke & Porter, 2016).

Morphologically, NWR exhibits several traits that dictate how a breeding program is managed. NWR inflorescence promote outcrossing in that female florets are located above male florets on the panicle. It is predominantly wind-pollinated, though Thieret (1971) noted bumblebees visiting male florets and collecting NWR pollen. Female flowers are receptive prior to the release of pollen on the same plant, and female flowers on a panicle do not become receptive at the same time (Kovach & Bradford, 1992b). This can make harvest management difficult as seeds do not ripen synchronously (Grombacher et al., 1997). Like other grasses, NWR exhibits tillering, but the tillers flower asynchronously with the main stem, further complicating appropriate harvest timing (Grombacher et al.,

1997). If early maturing seed shatters into the paddy, growers and researchers likely continue to indirectly select for shattering in their germplasm (Grombacher et al., 1997).

Native to the Great Lakes, NWR evolved a dormancy period to survive harsh winter conditions. NWR seed is considered to have intermediate recalcitrance, meaning seed is not desiccation tolerant. Because of this, seed must be kept at cool temperatures with high moisture content or it will not survive. Microbial growth is prevalent in high moisture storage conditions, which could be a reason for rapid decline in seed viability (Berjak & Pammenter, 2008; Makhathini, 2017; McGilp, Duquette, Braaten, Kimball, & Porter, 2020), ultimately making long-term storage difficult for recalcitrant/intermediate species like NWR. When dehydrated in warm temperatures, NWR seed may be able to be stored at a low seed moisture content (SMC), but must be carefully imbibed before cold stratification to allow the seed to break dormancy (Kovach & Bradford, 1992a). McGilp (2020) showed NWR seed could maintain high germination rate after up to 29 weeks in non-submerged, freezing storage, but a submerged, cold stratification period was necessary to ultimately break dormancy.

Dormancy in NWR is caused by an imbalance of plant growth hormones (Oelke et al., 1982). Abscisic acid, which promotes seed dormancy by preventing germination (Addicott et al., 1968), is found in higher levels in seeds that had not spent time in cold storage or had the dormancy period elapse (Albrecht, Oelke, & Brenner, 1979; Sondheimer, Tzou, & Galson, 1968). Much of the focus of the NWR breeding program revolves around managing uncontrolled pollen movement via outcrossing in-field and

improving storage quality through understanding dormancy and microbial growth in order to improve program and industry longevity of NWR as a cultivated crop.

Z. palustris: Domestication and Cultivation of a Species

Growing northern wild rice as a field crop was first suggested in 1852 and 1853 in Wisconsin and Minnesota respectively (Oelke et al., 1982). Domestication of the plant began in 1950 in Bass Lake, Minnesota, (Oelke et al., 1982) but evidence of seed dissemination throughout the Great Lakes region by Native groups has been documented long before 1950 (Hayes, Stucker, & Wandrey, 1989). Since the early 1960s, NWR has been cultivated widely in north central Minnesota, with commercial production today expanding to California, Wisconsin, Idaho, and Oregon (Oelke & Schreiner, 2007). The vast majority of cultivated NWR in the United States is produced in Minnesota and California (Zepp et al., 1996), with Minnesota accounting for approximately half of US NWR production in 2018 (Minnesota Cultivated Wild Rice Council, personal communication). At its peak in 1991, approximately 19,500 acres of NWR were cultivated in MN, up from a relatively low 11,500 acres grown in 2018 (Tuck, 2019) but acreage varies year by year.

NWR growers attempt to mimic the natural life cycle while maximizing crop and paddy efficiency. Therefore, cultivation practices differ slightly than lake or river harvested NWR. Cultivated NWR seed is drilled into soil or broadcast across paddies and then mechanically harvested. After harvest, grain is sent to a processor where it is cured to complete ripening, roasted to dry the seed and enhance flavor, hulled, scarified to remove

the bran layer, graded for packaging and end-use, and then cleaned of remaining debris like hulls. In 2018, 17.8 million pounds of green wild rice was processed to yield 9.0 million pounds of finished grain (Tuck, 2019). It is processed and sold as a dry grain, and is used as a component of rice mixes, granola bars, and other plant-based proteins/meat alternatives (Oelke, 1993; Zhai, Lu, Zhang, Sun, & Lorenz, 2001). In Minnesota, local butchers use wild rice in brat and burger recipes to enhance flavors and textures (Oelke & Boedicker, 2000; Rivera, Addis, Epley, Asamarai, & Breidenstein, 1996). Nutritionally, NWR is a good source of protein, fiber, vitamins, and minerals (Oelke & Schreiner, 2007) and has also been found to have cholesterol-lowering and antioxidant effects (Surendiran, Alsaif, Kapourchali, & Moghadasian, 2014). Compared to white rice, NWR has more than twice the protein per serving, twice the dietary fiber, nearly twice the potassium, and six times the zinc (Surendiran et al., 2014; Yu et al., 2020).

NWR has historically been grown on poorly draining, low-lying land, with high initial costs being incurred to build dikes/paddies. This land is poorly-suited for alternate crops making disease, weed, and insect management difficult as pest life cycles are not disrupted via crop rotation or fallow periods. Best management practices for NWR were published in 1997 and updated in 2006. There are several important diseases of cultivated NWR. Fungal brown spot (FBS), caused by *Bipolaris oryzae*, is the most prevalent and the most damaging disease (Johnson & Percich, 1992). *B. oryzae* also causes disease in white rice, and was the causal agent of the Bengal famine in 1943 wherein approximately two million people died of starvation (Padmanabhan, 1973). When disease is initiated at booting (PPS 4), FBS can cause yield losses as high as 74% (Kohls, Percich, & Huot,

1987). While there are no NWR cultivars that are fully resistant to FBS, variation in disease severity have been noted, indicating that there are opportunities to select for increased resistance (Grombacher et al., 1997). Other diseases commonly found in NWR include spot blotch (*Bipolaris sorokiniana*), zonate eyespot (*Drechslera gigantean*), and leaf sheath and stem rot (multiple pathogens including *Nakataea oryzae* and *Ceratorhiza hydrophila*), but they tend to cause fewer yield losses and are considered less economically relevant. Fungicide use is the main form of control, with propiconazole and azoxystrobin providing the best control when applied appropriately (Castell-Miller, Schlatter, & Samac, 2021). Utilizing several modes of action is essential to reducing resistance of the pathogen to the fungicide. The NWR integrated disease management strategy includes the reduction of infected plant residue biomass (Brantner, 1995), fall flooding, and one to two fungicide applications per growing season (Castell-Miller et al., 2021). Pesticides are applied aerially via plane.

Yield losses to weed competition are also well documented. Broadleaf weeds are more easily controlled chemically compared to grass weed species. Dense common waterplantain (*Alisma trivale* Pursh) growth for 9 weeks of the growing season correlated to a 50% yield reduction (Ransom & Oelke, 1982). In addition to chemical control, fall flooding has been shown to control common waterplantain (Ransom & Oelke, 1983). The wild rice worm, *Apamea apamiformis* (Guennee), is the most important insect pest of wild rice (Peterson et al., 1981).

NWR's cultivation as a lowland crop has made management different from other upland crops, but this careful management has been essential to NWR's success as a

commodity. In 2018, the NWR industry generated $58.4 million of economic activity. As a cultivated crop, ~90% of NWR grain is exported globally. The EU is a major importer of Minnesota grown NWR, and changes to import laws have made some aspects of NWR pest management more difficult. By making residue thresholds more stringent on imported grains, NWR growers have had to find new ways to control rice worm. The Interregional Research Project No. 4 (IR-4) program has been instrumental in approving new insecticides that control rice worm that are still in compliance with the standards of NWR's most economically important importer.

Despite cultivation beginning in 1950, a dedicated research/breeding program was not developed until 1971. The University of Minnesota NWR breeding program preliminarily began in 1963 when Erwin Brooks and Paul Yagya grew seeds on the St. Paul campus that were collected from natural stands. It was from this original seed lot that Yagya, along with Algot Johnson, developed the first non-shattering variety, 'Johnson,' despite no longer holding ties to the university. The Department of Agronomy and Plant Genetics, where the NWR breeding program is currently housed, continued to pursue funding opportunities for NWR and were finally awarded funds in 1971. Ervin Oelke, an agronomist by training, using selections donated from private researchers as well as growers, maintained germplasm in St. Paul until a breeder could be hired. The first full-time official breeder, Anson Elliot, was hired in 1972. It was shortly after that that paddies were constructed at the North Central Research and Outreach Center in Grand Rapids, MN, where all of the breeding program field research is conducted on NWR today. The first variety created by the breeding program, 'Netum,' was released in 1978. Historically, the

focus of the breeding program has been to reduce shattering, develop resistance to common diseases, reduce lodging, and breed for earlier maturing varieties that mature uniformly. These goals still remain the top priorities today, with extra emphasis being placed on shattering resistance and disease resistance. Fifteen varieties of NWR have been released in the state of Minnesota, nine of which were released by the breeding program—the remaining have been developed by growers (Oelke & Schreiner, 2007). Considerable progress in the program has been made, considering the short tenure of NWR as a cultivated crop. Comparatively, dedicated maize and soybean breeding programs at the University of Minnesota were established in 1915 and 1946 respectively (University of Minnesota. Department of Agronomy and Plant Genetics & Minnesota Agricultural Experiment Station, 2000).

Pollen Travel and Pollen-Mediated Gene Flow

Gene flow is the transfer of genetic material from one population to another, typically occurring within species but occasionally between species (Slatkin, 1985). Understanding gene flow is an important part of conserving genetic resources and population preservation (Fénart, Austerlitz, Cuguen, & Arnaud, 2007). It may be seed-mediated, pollen-mediated, or vegetative propagule-mediated, but because of spatial restrictions with seed dispersal, gene flow is largely dependent on pollen movement (Mallory-Smith, Hall, & Burgos, 2015; Scheepens, Frei, Armbruster, & Stöcklin, 2012). Due to the movement of pollen via wind, water, and insect pollinators, pollen-mediated gene flow (PMGF) occurs in almost all flowering plants (Ellstrand, Prentice, & Hancock, 1999; Glover, 2002; Mallory-Smith & Zapiola, 2008). The frequency of gene movement

depends on several factors that include population reproductive biology, pollen viability, species' mechanism of pollen dispersal, and breeding system (Loveless & Hamrick, 1984; Mallory-Smith et al., 2015). PMGF is also heavily influenced by population size, structure, and proximity (Ennos, 1994; Heywood, 1991). In both maize (*Zea mays*) (Messeguer et al., 2006) and common waterhemp (*Amaranthus rudis* Sauer) (Sarangi et al., 2017), evidence of pollen-mediated gene flow via wind was found within the first 50 m surrounding pollen donor plants but then dropped off considerably. Quantifying the breadth and depth of PMGF in outcrossing species is important to maintaining genetic diversity as well as maximizing efficiency in breeding efforts.

The consequences of gene flow may be beneficial, neutral, or detrimental (Ellstrand et al., 1999), but PMGF in outcrossing species often represents the movement of unwanted alleles from one population to another. This movement can affect varietal purity and cause crop-to-wild or weedy relative gene flow (Ellstrand, Barrett, Linington, Stephenson, & Comai, 2003). Crop-to-weed gene flow could have detrimental impacts if traits that confer improved fitness or stress tolerance "escape" into weedy relatives, ultimately making their control even more difficult (Hancock, Grumet, & Hokanson, 1996). For example, a red pigmented white rice cultivar was planted in Japan to facilitate weeding, preventing weedy wild rice from persisting in fields; after several seasons, the weed population accumulated the pigmentation alleles at high frequency (Oka, 1959). Gene flow from transgenic crops is carefully monitored, as the movement of a transgene into a non-GM cultivar or weedy relative is an environmental concern. Transgene migration affects varietal purity and can make management more difficult and more expensive. Evidence of hybridization between

transgenic canola (*Brassica napus*) and weedy *Brassica rapa* has been reported in Canada (Warwick et al., 2003); as well as between creeping bentgrass (*Agrostis stolonifera*) and natural populations of other *Agrostis* species (Reichman et al., 2006; Watrud et al., 2004). In the case of transgene escape into non-GM cultivars, the maintenance of certified seed populations involves genetic testing post-harvest to determine varietal purity. Techniques utilized to monitor transgene escape include marker-based characterization of hybridization and transgene introgression, marker-based migration rate estimation, and the analysis of variation in transgene frequency to make inferences on migration and selection (Schoen, Reichman, & Ellstrand, 2008). For NWR, crop-to-wild gene flow of non-shattering genes could introduce common agronomically-selected alleles to natural stands, and wild-to-crop gene flow could re-introduce shattering genes into domesticated populations.

The dispersion of pollen can depend on numerous factors including pollen viability, flowering synchrony, weather conditions, and surrounding vegetation (Gliddon, Boudry, & Walker, 1999; Shivanna, Linskens, & Cresti, 1991; Thompson, Squire, Mackay, Bradshaw, & Crawford, 1999). In particular, weather conditions, such as wind speed, wind direction, and relative humidity, significantly affect pollen travel trends in wind-pollinated species (Born, Le Roux, Spohr, McGeoch, & Van Vuuren, 2012; Latta & Mitton, 1999; Steinitz, Troupin, Vendramin, & Nathan, 2011). Studies have shown that pollen concentration in the air decreases significantly on rainy days, as rain washes pollen grains from the air (Hart, Wentworth, & Bailey, 1994; Scott, 1970). Despite this heavy environmental influence, most models predict the rapid dissipation of pollen densities

following release from the source (Messeguer et al., 2006; Sarangi et al., 2017). For breeders and conservationists alike, an understanding of pollen travel trends for species of interest can aid in eliminating or reducing unwanted pollen-mediated gene flow.

The movement of pollen can be monitored using a wide range of techniques. Traps to track the physical movement and deposition of pollen, mostly commonly sedimentation and filtration traps, have been used across a range of species (Kearns & Inouye, 1993; Mullins & Emberlin, 1997). Other trapping methods involve the use of marked or tagged pollen (Moon, Halfhill, Hudson, Millwood, & Neal Stewart, 2006). Specifically tracking PMGF requires the use of morphological traits, such as kernel color (Bannert & Stamp, 2007; Hanson, Mallory-Smith, Shafii, Thill, & Zemetra, 2005), cone size (Zobel, 1951), and leaf hair density (Hardig et al., 2000), or molecular markers, which can track specific genes (Ouborg, Piquot, & Van Groenendael, 1999). Molecular markers, in particular, have played an important role in tracking gene movement from genetically modified (GM) crops to non-GM varieties, landraces, and weedy relatives (Ma, Subedi, & Reid, 2004; Pla et al., 2006; Watrud et al., 2004). Manasse (1992), utilized an anthocyanin expressing *Brassica campestris* (Chinese cabbage) variety to track gene movement into an anthocyanin suppressing variety. Progeny that had been harvested from the anthocyanin suppressing variety but expressed anthocyanin represented the movement of the anthocyanin expressing genes into that population. Utilizing an easily-discernible morphological trait will allow for efficient data collection when monitoring PMGF.

History of Pollen Studies in NWR

It is absolutely imperative to understand pollen movement in NWR for two reasons. First, it is integral to the development of a successful NWR breeding program, as the permanent paddy structures leave little space for isolation. It is also essential to the preservation of natural stands of NWR that can grow in close proximity to cultivated NWR across the state of Minnesota. The set of traits lost in domestication like shattering, non-compact growth habit, photoperiod sensitivity, and seed dormancy, also known as domestication syndrome (Hammer, 1984) still persist in natural stands. The movement of shattering genes into a cultivated NWR population represents loss of yield, while the movement of non-shattering genes into the natural stands could represent the loss of fitness. Should separate (cultivated and natural) populations find the opportunity to hybridize, the results could be unfavorable to both populations. Understanding pollen travel and pollen-mediated gene flow allows growers to become better stewards of their domesticated plants.

An important step in the development of a successful breeding program for an outcrossing species is determining the minimum distance allowable between two populations while maintaining genetic purity. Much of the early research in the program was motivated by this idea. Pollen trapping occurred very early on in the history of the breeding program. The first pollen trapping study was reported on in 1973 (Elliott, 1974). They found that pollen grains were able to travel upwards of 150 yards (based on collecting 1.5 pollen grains/0.1 cm^2 in the trap), and therefore extrapolated that an isolation distance of 250 yards may be a realistic guideline (Elliott, 1974). The use of cloth barriers or pollen tents were first mentioned in 2000 to reduce pollen travel and improve population isolation

(Porter, Boedigheimer, & Schumer, 2000), but are currently not used in-paddy in the program. Pollen trapping was then re-tested in 2002 when Joanna Cregan explored pollen travel. Her pollen trapping results showed that temperature, relative humidity, and wind speed affected pollen release (Cregan, 2004). While results varied in each year of the study, in general pollen release was highest from 20-27 °C, 50-70% RH, and 7.5-9 kph wind speed (Cregan, 2004). This was specific to the farm at which the data was collected and does not reflect pollen release of NWR in totality.

Pollen viability plays a vital role in understanding the scope of pollen-mediated gene flow. Even if pollen grains are able to travel for miles, if they are no longer viable at deposition, pollination and thus fertilization will not occur. Research conducted in 1988 showed that NWR pollen was most viable between 0 and 2 hours after anthers had opened, with a rapid drop off in viability after two hours (Page & Stucker, 1990). Additionally, Page found that hour of pollination had no effect on seed set and thus pollen was not more viable at a certain time of day. Time of day is not as important as pollen quality (Brown & Shands, 1956; Hallauer & Sears, 1966). Stigmas were receptive for several days and therefore were not limiting factors in seed set, however stigma receptivity and pollen viability were likely affected by temperature and relative humidity.

As previously cited in other crops, phenotypic traits can be used effectively to quantify the effects of pollen-mediated gene flow. Several populations have been developed and several traits explored for the purposes of tracking gene flow in NWR. In 1978, selections were made from a ‘Johnson’ population to create a breeding population that had white flowers, easily discernible from the typical/dominant purple flower color,

with the intention of utilizing it to determine population isolation distance. Once it was determined that the population was still segregating for flower color, it was unusable as a receptor population to track gene flow. Over the last three decades, this population has been kept isolated and carefully maintained. In 1974, research began to determine how leaf sheath and collar color were inherited for use in tracking gene flow/use as phenotypic markers. In his master's research, Gary Perlinger showed that colored plant sheaths were controlled by a gene being present at each of two loci (Elliott, 1976; Perlinger, 1976). Seed set, and thus pollen travel, has also been evaluated in a bottlebrush (BB) population (Cregan, 2004), which has a compact male floret morphology linked to an uncharacterized male sterility gene (Grombacher et al., 1997; Stucker, Linkert, Palm, & Hernandez, 1984). Widely cited to demonstrate concerns regarding pollen-mediated gene flow in NWR, Cregan (2004) reported that NWR pollen could travel at least 3200 m. However, the linkage between the BB trait and male sterility can often be broken and pollen-producing BB plants were identified in the study, leading to seed set (contamination) and the conclusion that the BB trait is not adequate for NWR pollen travel studies. Due to the recessive nature of the white flower trait, a white flower NWR population should work well for pollen travel studies and the linkage between various plant tissue colors may allow for expedited selection before flowering.

Basic research on pollen travel, pollen viability, and pollen-mediated gene flow has been conducted throughout the history of the NWR breeding program. However, as technology improves and scientific consensus changes, initial pollen studies should be

reviewed and built upon to maximize the understanding of the impact of pollen movement and gene flow.

Seed Biology

Seed desiccation tolerance has important implications for seed longevity, and seed quality. Orthodox seeds are able to survive drying and freezing, while recalcitrant seeds are unable to survive drying and freezing temperatures (Roberts, 1973). A third class of desiccation tolerance has since been defined. Seeds with intermediate recalcitrance do not survive complete drying, but are able to tolerate lower seed moisture contents (SMC) and lower storage temperatures than recalcitrant seed (Ellis, Hong, & Roberts, 1990; Hong & Ellis, 1996). In addition to NWR, other species with economic importance and intermediate seed recalcitrance include coffee (Ellis et al., 1990), papaya (Ellis, Hong, & Roberts, 1991), hickory (Raven, 2004), and citrus (Raven, 2004).

Orthodox seed may be stored for up to 25 years when stored in conditions recommended by the IPGRI (Hong, Linington, & Ellis, 1996). Additionally, some species are able to survive storage in liquid nitrogen, further extending their longevity (Stanwood & Bass, 1981; Stanwood, 1985). Oppositely, the moisture in intermediate and recalcitrant seed reduces the scope for modifying the storage environment as ice nucleation damages the seed, leading to difficulty in improving storage conditions beyond short periods (King & Roberts, 1979). Instead, other methods of germplasm preservation must be utilized. *In vitro* preservation (Engelmann, 1991), embryo rescue, pollen storage, and cryopreservation (Stanwood, 1985) are viable options of *ex situ* conservation of recalcitrant species. Their

usefulness in NWR is still not fully understood, but previous tissue culture experiments were unsuccessful as plant regeneration did not regularly occur from callus (Johnson & Percich, 1992; Percich, Zeyen, Johnson, & Malvick, 1990). Cryopreservation of embryos may be a viable option for NWR, but success varied based on other factors like moisture content at freezing and light level during recovery (Touchell & Walters, 2000).

NWR's unique seed physiology makes long term storage difficult. Seed does not maintain its viability for more than one year. This has negative implications for conservation, *ex situ* seed storage, as well as for annual rate of genetic gain. For all crops, improving or maintaining seed longevity is important for high quality planting material, pre- and post-growing season food availability, as well as maintaining genetic resources in seed banks.

Seed dormancy causes a delay of germination even when favorable growth conditions are met. Environmental cues that lead to germination include light level and fluctuating temperature, in addition to moisture level/imbibition of water (Baskin & Baskin, 2004; Presotto, Poverene, & Cantamutto, 2014; Vandelook, Van de Moer, & Van Assche, 2008). Dormancy is determined by genetic factors with heavy environmental influence (Graeber, Nakabayashi, Miatton, Leubner-Metzger, & Soppe, 2012). There are five accepted classes of dormancy recognized in the literature, which include physiological, morphological, morphophysiological, physical, and combinational dormancy (Baskin & Baskin, 2004). Physiological is the most common kind (Baskin & Baskin, 2004) and involves plant growth hormone imbalance, namely abscisic acid and gibberellic acid. Abscisic acid (ABA) regulates numerous aspects of plant growth, including inhibiting germination thus playing

an important role in dormancy (Addicott et al., 1968; Hilhorst, 1995; Kermode, 2005). In contrast, gibberellic acid (GA) promotes growth by inducing mitotic division and promoting cell elongation (Derkx, Vermeer, & Karssen, 1994). Recent molecular analyses support a theory in which GA metabolism is oppositely regulated to that of ABA (Yamaguchi, 2008). This was also shown in NWR (Albrecht et al., 1979). In NWR, a waxy seed coating (physical dormancy) persists as well as physiological dormancy, complicating efforts to break dormancy sooner to increase annual genetic gain.

Microbial Growth and Control in Seed Storage

Microbial growth in orthodox seed due to improper storage conditions may lead to a decrease in seed quality. While the seed/storage microbiome is complex in that many microbes exist in the system without causing harm, storage fungi have been documented that damage seeds and lead to human or animal illness (Meronuck, 1987). A common fungus that causes problems in storage, *Aspergillus flavus*, produces aflatoxin which causes aspergillosis when inhaled and aflatoxicosis when ingested (Amaike & Keller, 2011). Though plant infection occurs pre-harvest, improper storage conditions exacerbate aflatoxin contamination (Amaike & Keller, 2011). Aflatoxin does not cause yield loss directly, but the economic loss due to *A. flavus*/aflatoxin contamination contributes heavily to losses associated with mycotoxin contamination in general, which is estimated at 1 billion dollars (Science, 2003). Microbial growth in the submerged/hydrated storage of recalcitrant/intermediate species is a contributing factor to their short storage life (King & Roberts, 1979). One study identified seed-associated microbes of seven recalcitrant

species, and found *Fusarium* was the predominant genus (Mycock & Berjak, 1990). More recently, Makhathini (2017) conducted a more comprehensive study of the seed microbiomes of three recalcitrant tree and bush species: *Protorhus longifolia*, *Trichilia dregeana*, and *Garcinia livingstonei.* Ultimately, no such studies evaluating the effect of the seed microbiome on seed quality and germinability have been conducted using recalcitrant seed from aquatic species.

Controlling microbial growth in storage can be difficult. Seed treatments improving seed longevity, seedling health, and overall storage quality have been used for centuries. In 1651 Samuel Hartlib noted that the brining and subsequent liming of cereal grains reduced the prevalence in smut as well as increased yield in the subsequent growing season (Hartlib, 1651; Smith & Secoy, 1976). Today, fungicidal seed treatments (FSTs) are used extensively in modern conventional farming practices (Lamichhane, You, Laudinot, Barbetti, & Aubertot, 2020; White & Hoppin, 2004). The first commercial FSTs were organomercurial dusts, first released in 1927, deployed to control smuts and bunts of cereals (Taylor, Guirgis, & Stewart, 1969). In the 1940s, Thiram was developed as a seed treatment to expand the spectrum of disease control (Peacock, 1978). Today, many different FSTs work to maintain seed and seedling viability at planting in many crops, providing protection against many common seedling diseases.

FST use in recalcitrant species has not been widely studied. In citrumelo, FST use did help to control storage pathogens and seed quality was maintained in storage (Silva, Guimarães, von Pinho, & Abreu, 2011). A native Brazilian palm tree species showed increased germination and decreased fungal growth when treated with an FST (Lacerda et

al., 2016). Makhatini (2017) showed that some chemical fungicides were effective at reducing the growth of fungal isolates, initially collected from three recalcitrant tree species native to Africa. The study also showed that when one fungicide was used as a seed treatment it improved seedling vigor, when used in combination with a surface decontaminant (NaOCl) and seed encapsulation (Makhathini, 2017). Implications of FST use on annual, aquatic/recalcitrant crop species are not fully explored in the literature. However, general antimicrobial treatments are used commonly in commercial and research agricultural settings. Sodium or calcium hypochlorite (bleach) has been used as an antimicrobial seed treatment to reduce the pathogenic load that leads to a decrease in seed quality and an increase in human illness (Sauer, 1986; Schultz & Gabrielson, 1986; Stewart, Reineke, Ulaszek, & Tortorello, 2001). Blanchard and Hanlin (1973) showed 80-92% of microbial elimination when seed was treated with propylene oxide. Antimicrobial treatments also allow for the surface sterilization of seed in protocols that improve *ex situ* storage like embryo rescue and tissue culture, rather than just storing seed (Engelmann, 2012; Farzana, Palkadapala, Meddegoda, Samarajeewa, & Eeswara, 2008; Mundt & Hinkle, 1976; Nower, 2013).

The seed microbiome is complex as it includes the microbes found within the seed (endophytic microbes), as well as the microbes on the surface of the seed coat (epiphytic microbes) and the surrounding area. Seed microbiome composition appears to be genotype specific (Adam, Bernhart, Müller, Winkler, & Berg, 2018; Rybakova et al., 2017), but variability is even seen between individual plants within a monoculture cropping system (Hamid et al., 2017). Changes to microbiome composition may occur throughout the

storage period or growing season. For example, when seeds are surface sterilized, microbes within the seed may take the space previously occupied by the outer microbes (Blanchard & Hanlin, 1973). This could have positive or negative implications. Healthy seeds are considered aseptic (Baker & Smith, 1966; Pande, Rao, & Sharma, 2007; Zheng, Lopisso, Eseola, Koopmann, & von Tiedemann, 2019).

The composition of the seed microbiome depends on many factors, like weather, soil composition, nutrient availability, and water, the individual extent to which each of these have on the plant remains unclear (Buyer, Roberts, & Russek-Cohen, 1999; Hacquard, 2016). This can make generalizing the composition of the microbiome difficult, but the ability to identify the "core" microbes that appear regardless of year, genotype, and location has important implications for the development of agricultural products (Barret et al., 2015; Parnell et al., 2016). Seed-associated microbes may be beneficial, harmful, or they may have no effect on the seed at all. Currently, there are many documented seedborne pathogens noted in the literature. In small grains, *Fusarium* spp., *Pythium* spp., and *Rhizoctonia* spp. are the most common and detrimental to seed and seedling health. There is also evidence in the literature of beneficial microbes acting to help with germination (Alavi, Starcher, Zachow, Müller, & Berg, 2013; Jacquemyn, Waud, Merckx, Lievens, & Brys, 2015), break dormancy (Delgado-Sánchez, Ortega-Amaro, Rodríguez-Hernández, Jiménez-Bremont, & Flores, 2010), or suppress pathogenic microbes (Weller, Raaijmakers, Gardener, & Thomashow, 2002).

Methods to identify the seed microbiome began by isolating and purifying microbes from seed and other plant tissues. This has been used extensively in the literature to

identify members of plant microbiomes with varying success (Cottyn et al.; Granér, Persson, Meijer, & Alström, 2003; Kanivets & Pishchur, 2001; Kremer, 1987; Mundt & Hinkle, 1976; Wallace & Lochhead, 1951). Kremer (1987) identified seed-associated microbes by surface sterilizing and streaking seeds on agar and purifying microbes that arose. As technological advances have been made in molecular biology, metagenomics analysis is used more frequently today to identify the composition of the microbiome with more specificity and reduced error. Metagenomics analysis involves high-throughput sequencing of DNA in a microbial community, enabling high-resolution taxonomic and functional characterization (Loman et al., 2012; Simon & Daniel, 2011).

Ultimately the preservation of NWR, cultivated and natural, is essential. The complex seed physiology that includes intermediate recalcitrance, the presence of a dormancy period, as well as rampant microbial growth in storage, will continue to make conservation of genetic resources difficult, if not impossible. Improvement of storage conditions is essential to the survival of NWR as a species.

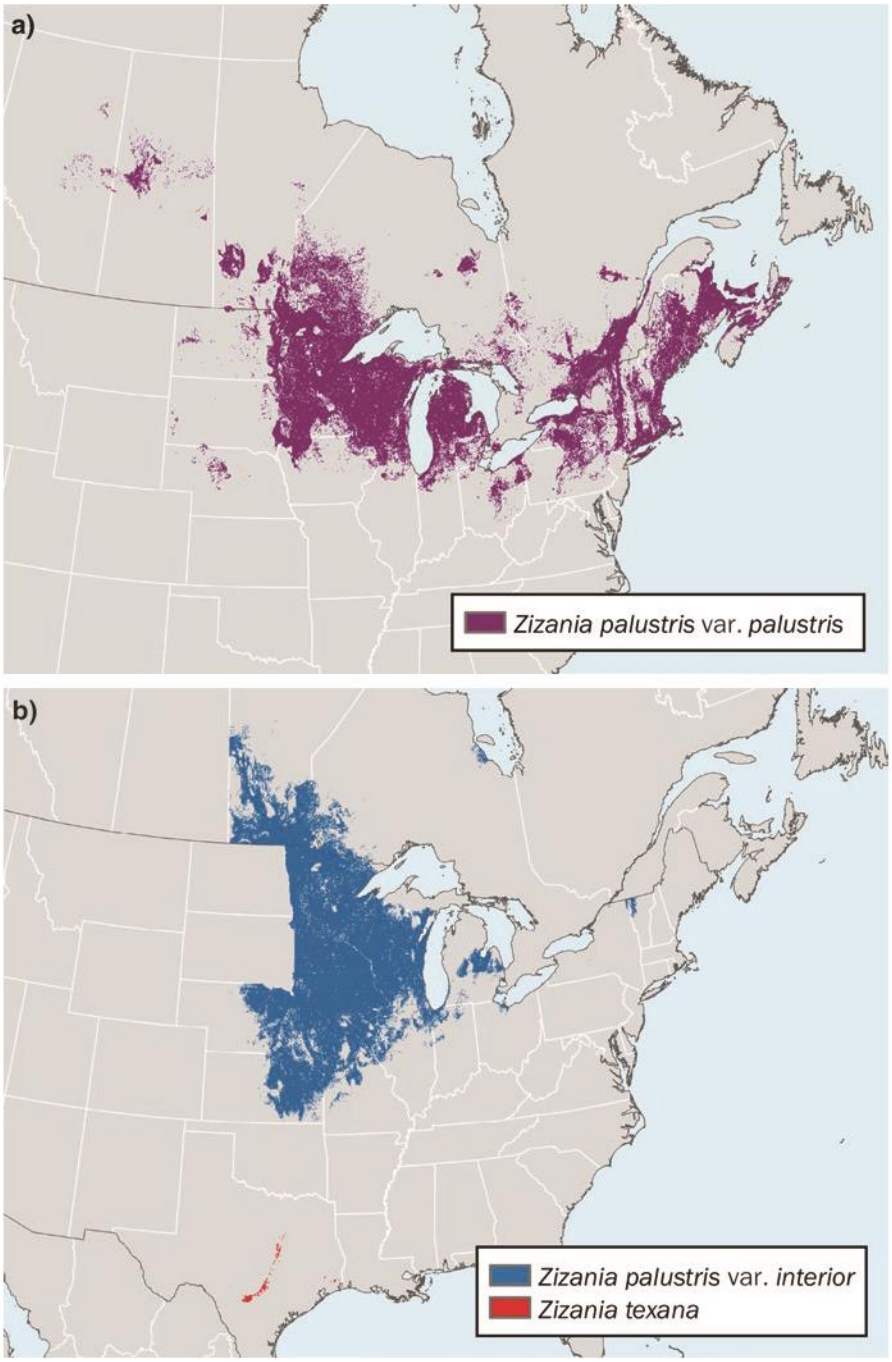

Figure 1 Northern Wild Rice Species Distribution. Modeled potential distribution of a) *Zizania palustris* var. *palustris* and b) *Z. palustris* var. *interior* and *Z. texana* based on climatic and soil similarities. Adapted from Porter, 2019.

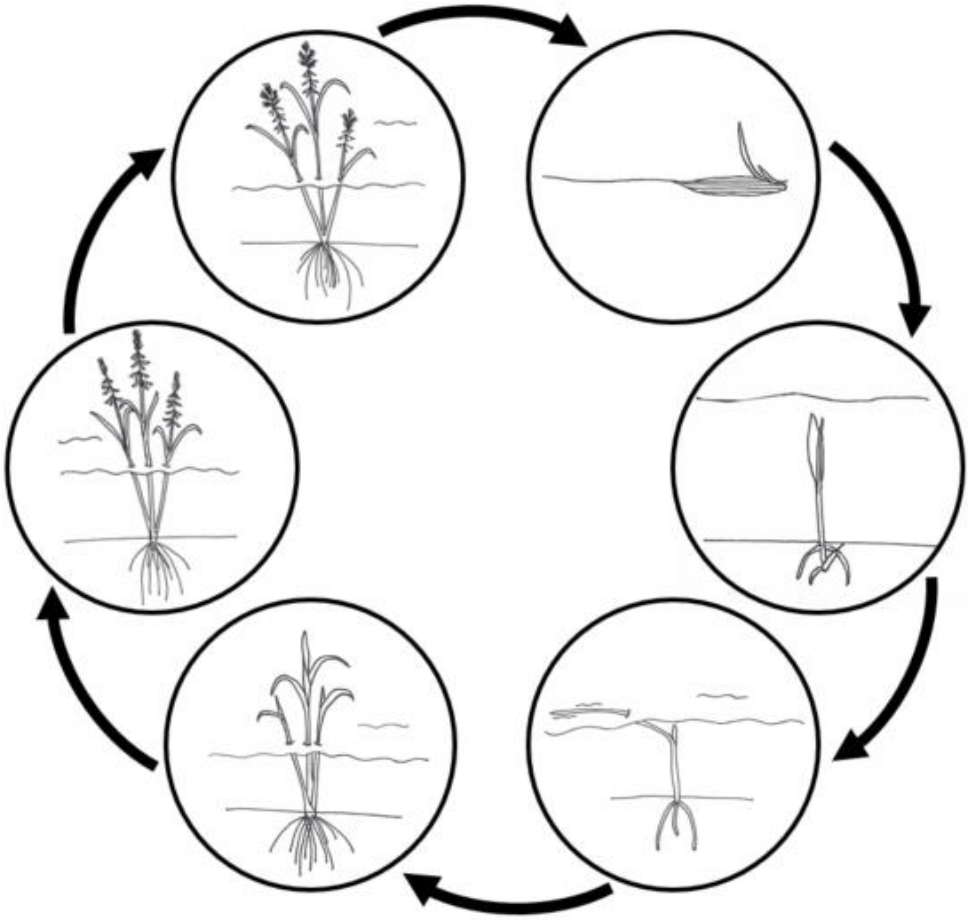

Figure 2 Northern Wild Rice Life Cycle. Submerged seeds germinate in the spring and grow towards the surface of the water. As the growing season elapses, plants begin to grow tillers. Flowering occurs in mid-summer, with seed reaching full maturity in the fall. Mature seed shatters back into the body of water to continue the cycle after an overwintering period.

Chapter 2 – Evaluation of a Recessive Male Floret Color in Cultivated Northern Wild Rice (*Zizania palustris* L.) for Pollen-Mediated Gene Flow Studies

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Abstract

Northern wild rice (NWR; *Zizania palustris* L.) is a wind-pollinated, annual, aquatic grass that grows naturally in the Great Lakes Region (GLR) of the United States and Canada, and is also cultivated in flooded paddies, predominantly in California and Minnesota. A better understanding of pollen-mediated gene flow is needed within the species for both conservation and breeding efforts as cultivation occurs within the species' natural range and spatially-isolated, paddy structures are limited within breeding programs. Widely cited pollen travel research in NWR demonstrated that pollen could travel at least 3200 m. However, a population segregating for male sterility was used as the pollen recipient in the study and was determined to not be adequate for NWR pollen travel studies. Here, we present the characterization of a recessive white male floret (WMF) population in contrast to the dominant, purple male floret (PMF) color of cultivated NWR along with estimates of pollen-mediated gene flow in a cultivated paddy setting. Studies conducted in 2018 and 2019 revealed that the primary amount of pollen-mediated gene flow occurred within the first 7 m from the PMF donor source with no gene flow detected past 63 m. These results suggest that the likelihood of pollen-mediated gene flow between cultivated NWR and natural stands remains low. We also identified a strong linkage between male floret,

auricle, and culm color. This study demonstrates that the WMF trait is an excellent candidate for use in pollen-mediated gene flow studies in NWR.

Introduction

Pollen-mediated gene flow in outcrossing species often represents the movement of unwanted alleles from one population to another. This movement can affect varietal purity and cause crop-to-wild or weedy relative gene flow (Ellstrand et al., 2003). The dispersion of pollen can depend on numerous factors including pollen viability, flowering synchrony, weather conditions, and surrounding vegetation (Gliddon et al., 1999; Shivanna et al., 1991; Thompson et al., 1999). In particular, weather conditions, such as wind speed, wind direction, and relative humidity, significantly affect pollen travel trends in wind-pollinated species (Born et al., 2012; Latta & Mitton, 1999; Steinitz et al., 2011). Despite this heavy environmental influence, most models predict the rapid dissipation of pollen densities following release from the source (Messeguer et al., 2006; Sarangi et al., 2017). For breeders and conservationists alike, an understanding of pollen travel trends for species of interest can aid in eliminating or reducing unwanted pollen-mediated gene flow.

The movement of pollen can be monitored using a wide range of techniques. Traps to track the physical movement and deposition of pollen, mostly commonly sedimentation and filtration traps, have been used across a range of species (Kearns & Inouye, 1993; Mullins & Emberlin, 1997). Other trapping methods involve the use of marked or tagged pollen (Moon et al., 2006). Specifically tracking pollen-mediated gene flow requires the use of morphological traits, such as kernel color (Bannert & Stamp, 2007; Hanson et al., 2005), cone size (Zobel, 1951), and leaf hair density (Hardig et al., 2000), or molecular

markers, which can track specific genes (Ouborg et al., 1999). Molecular markers, in particular, have played an important role in tracking gene movement from genetically modified (GM) crops to non-GM varieties, landraces, and weedy relatives (Ma et al., 2004; Pla et al., 2006; Watrud et al., 2004).

Northern wild rice (NWR), *Zizania palustris* L., is an annual, wind-pollinated aquatic grass native to the Great Lakes Region of North America. It is a protogynous, monoecious species and its female florets are located above male florets on the panicle. Cultivation of the species began in Minnesota in the 1950s (Oelke et al., 1982) and has since become a high-value specialty crop in major markets (Tuck, 2019). Breeding strategies and varietal development are largely based on phenotypic recurrent selection within open-pollinated populations (Grombacher et al., 1997). Spatially-isolated, permanent paddy structures are limited within breeding programs, necessitating a better understanding of pollen travel trends, especially within short distances, to maintain the genetic purity of breeding lines (Baltazar et al., 2015; Ireland, Wilson, Westgate, Burris, & Lauer, 2006). Additionally, cultivation occurs within the species' center of origin, where the conservation of natural stands in lakes and streams is imperative to the maintenance of genetic diversity and delicate ecosystems. NWR is considered an indicator of overall ecosystem health, providing food and habitat for a variety of wildlife (Fannucchi, 1983; Rogosin, 1954). Therefore, an understanding of potential gene flow from natural stands to cultivated paddies and vice versa is an integral aspect of pollen travel research in NWR.

Initial pollen travel studies in NWR found diurnal pollen release patterns, similar to those in corn and other grass species, with the highest pollen concentrations occurring

between 1200-1700 hours, 20-23 °C, 50-60% relative humidity, and wind speeds of 7.5-9 kph (Cregan, 2004). Previous NWR pollen travel research also included the utilization of a bottlebrush (BB) population (Cregan, 2004), with a compact male flower morphology, linked to an uncharacterized male sterility gene (Grombacher et al., 1997; Stucker et al., 1984). Cregan (2004) reported that NWR pollen could travel at least 3200 m, a number that has been widely cited as validation of concerns regarding pollen-mediated gene flow in NWR. However, the BB trait is considered inadequate for NWR pollen travel studies due to the somewhat weak linkage between the BB trait and male sterility, leading to pollen-producing BB plants and resulting seed set (contamination). In this study, we confirm the utilization of the male floret color trait, either a recessive white male floret (WMF) or a dominant purple male floret (PMF), to estimate pollen-mediated gene flow in a cultivated paddy setting. This study demonstrates that the WMF trait is an excellent candidate for use in pollen-mediated gene flow studies in NWR.

Materials and Methods

Plant Material. In this study, a homozygous recessive WMF population (Figure 3a) was utilized to estimate pollen-mediated gene flow in NWR. Multiple PMF populations (Figure 3b) were utilized as pollen donors to maximize the flowering synchronicity between WMF and PMF populations. The 2018 flowering period for the WMF population was from July 15th to August 15th and from July 25th to August 25th in 2019.

Weather Data. Wind speed (kph) and direction data were collected from the National Oceanic and Atmospheric Administration (NOAA) weather station located at the Itasca

County Airport in Grand Rapids, MN, approximately 5.3 kilometers from the trial. Air temperature (°C) and accumulated precipitation (mm) were collected from an automated weather station at the Grand Rapids U.S. Forest Research Service Lab in Grand Rapids, MN, approximately 0.8 kilometers from the trial.

Pollen Travel Experiments. Experiments were conducted at the University of Minnesota North Central Research and Outreach Center (NCROC) in Grand Rapids, Minnesota (47.2372° N, 93.5302° W, and 392 m elevation) at the cultivated NWR paddy complex, where individual paddies have 1 to 1.5 m raised dikes for irrigation purposes. In 2018, twenty 3 m x 6 m WMF plots with 3 m single rows spaced 0.38 m apart and 1 m alleys were planted at 15.7 kg ha^{-1}. These plots were planted 3 m south/southeast of a 24-plot (12 m x 41 m) PMF trial, which served as the purple pollen donor (Figure 4). At its farthest distance, a WMF plot was a maximum of 35 m from a PMF plot (north to south). In 2019, the experiment was expanded to include larger pollen travel distances and forty-four 3 m x 6 m WMF plots were planted with the northwestern-most plot consisting of multiple PMF genotypes (Figure 4). The maximum distance between a WMF plot and the PMF plot was 9 m, west-east, and 70 m, north-south. The experimental design for these experiments was largely dictated by the size and shape of paddies (~15-25 m wide and ~90 m long), which eliminated the possibility of planting a PMF plot in the middle of a large WMF plot to evaluate 360 degrees of pollen travel from the source. Instead, common summer wind patterns were utilized to establish dominant wind directions at the paddy complex, resulting in the PMF donor source being planted directly north of the WMF plots.

Paddies were amended with 16.7 kg ha^{-1} Environmentally Safe Nitrogen (ESN), 9.3 kg ha^{-1} urea, and 7.4 kg ha^{-1} potassium. Immediately following planting, paddies were flooded to 20 cm average water depth. Copper sulfate (1.13 kg ha^{-1} rate) was used to control algal growth and Aquabac (200G) (*Bacillus thuringiensis* subsp. *israelensis* - Becker Microbial Products, Coral Springs FL; 1.86 kg ha^{-1} rate) to control aquatic pests, specifically midge larvae. Plots were monitored daily for PMF plants in the WMF plots, which were rogued prior to pollen set. Individual WMF plots were harvested each year. Seed was then cleaned of other plant debris, weighed, and stored in the dark, at 3 °C, on water, for the duration of the seed dormancy period.

Progeny Testing. For both years of the study, ~50-100 stratified seeds per WMF plot were germinated on petri dishes lined with filter paper and hydrated with 10 ml of H_2O. The petri plates were placed at an ambient temperature of ~23 °C, under LabLink LED LabLights (PG LifeLink, Erlanger, KY), set to a 16-hr day length. After 10 days, 5 cm long seedlings were transplanted into individual 25 cm deep plant cones, submerged in 680 L aquaponics tanks. The soil in each cone was amended with 22.4 kg ha^{-1} N, in the form of urea, and 0.125 g iron chelate. Throughout growth and flowering, plants were monitored daily for culm, auricle, and floret color. Anecdotal evidence within the program suggests a potential link between the genes controlling these traits. In the spring of 2019, ~38 progeny plants per WMF plot, from the 2018 study, were evaluated. For the 2019 trial, only ~22 progeny plants per WMF plot were evaluated in the spring of 2020 due to the COVID-19 global pandemic.

Data Analysis. Monthly mean air temperature was calculated by averaging the daily maximum and minimum air temperature values. Cumulative precipitation was calculated by adding together the daily precipitation, in millimeters, for all days in a given time period. Analysis of wind speed and direction data was conducted using R 4.0.1 (R Core Team, 2020), and the OpenAir package (Carslaw & Ropkins, 2012) was used to generate wind roses with the use of ggplot2 and ggplotify (Wickham, 2016; Yu, 2020). Wind run, or the total distance that the wind traveled over a given timeframe, was calculated on a 24 hour basis by multiplying the average wind speed by the amount of time elapsed per data point. Data was collected hourly unless a shift in wind direction occurred, in which case, another data point was generated. In order to analyze differences in wind run per plot, the degree range for each of the 16 cardinal, ordinal, and inter-ordinal directions was superimposed onto each plot map and wind directions that conferred a high probability of wind traveling through pollen donor PMF plots to each WMF plot was determined. An empirical model of pollen-mediated gene flow was obtained by regressing the hybridization rate on the maximum distance from the PMF pollen source using the NLIN procedure in SAS statistical software package v9.4 (SAS Institute, 2013) similar to Schmidt *et al.* (2013). The negative exponential model: hybridization rate = $\alpha^{-\beta(\text{distance})}$ was used. The suitability of the model fit was verified using regression coefficients.

Percent hybridization per plot was calculated as the proportion of WMF plot progeny with purple male florets over the total progeny tested. To evaluate the effect of distance from the PMF source, the distance in meters from the closest edge of the PMF

plot(s) to the furthest edges of each WMF plot was measured and then pooled for each range of the experiment. Recombination frequencies between culm, auricle, and male floret color were calculated using parental (all white or all purple phenotypes) and recombinant class data from 2018. The number of plants in each recombinant class divided by the total number of observed parental phenotypes provided estimates of genetic linkage in centiMorgans (cM) between the three traits.

Results and Discussion

Weather Data. Throughout the growing period in 2018, the mean air temperature (°C) was generally warmer than the 20-year average for Grand Rapids, Minnesota but in 2019, it was cooler than the 20-year average (Table 1). Temperature differences between 2018 and 2019 were minimal, with the exception of May and August, when it was more than 2 °C cooler in 2019. During the 2018 and 2019 flowering periods (July 15th-August 15th and July 25th-August25th, respectively), weekly mean air temperatures varied widely. The last week of July and first week of August were peak flowering weeks during both years and had a mean air temperature of 20.0 °C and 17.9 °C in 2018, and 13.9 °C and 19.1 °C in 2019, respectively. In other Poaceae species, temperature is known to affect anther ripening. Warmer weather can confer higher concentrations of shed pollen (Hart et al., 1994), while low temperatures can reduce meiotic efficiency during anthesis (Zeng et al., 2017). Cregan (2004) found NWR pollen shed to be highest between 20 to 23 °C, which suggests warm, but not hot, temperatures improve pollen shed. It is possible that the low temperatures during the first week of peak flowering in 2019 decreased pollen shed.

However, more research regarding the relationship between temperature and pollen shed in NWR is needed to validate this conclusion.

Monthly precipitation (mm) in 2018 was similar to the 20 year average with the exception of the month of August, which was ~43% drier than the 20 year average (Table 1). In 2019, monthly precipitation levels were highly variable from month to month during the growing season and their 20 year averages. May, July, and August were drier in comparison, while June and September were wetter. When comparing 2018 and 2019, accumulation varied widely between the months. During the flowering periods, plots received a total of 12.96 mm of precipitation in 2018 and 78.22 mm in 2019. While the effect of precipitation on pollen travel in NWR has not been previously explored, studies have shown that pollen concentration in the air decreases significantly on rainy days, as rain washes pollen grains from the air (Hart et al., 1994; Scott, 1970). In 2018, there were 11 days during the 30-day flowering period with rainfall, however only 3 of those days accumulated to more than 1 mm. In 2019, there were 17 days of recorded rainfall during the 30-day flowering period but only 11 with more than 1 mm.

Wind direction and speed are primary drivers of pollen travel in wind-pollinated species. In 2018, winds originated predominantly from the northwest and southeast, while winds in 2019 primarily arose from the south (Figure 5). In 2018, ~33% of the wind came from the northwest (NW, NNW, WNW), and ~31% came from the south (S, SSE, SSW). In 2019, ~23% of the wind came from the northwest, while ~46% came from the south (Figure 5). Mean wind speeds were similar between years with speeds reaching 7.9 kph in 2018, and 8.2 kph in 2019. Both years had similar amounts of calm air during the flowering

periods (38.4% and 36.4%, respectively). Both years also had similar frequencies of high speed wind. During the flowering period in both years, approximately 1% of the wind that blew was characterized as a "fresh breeze," defined as 29-38 kph on the Beaufort scale. In 2018, 7% of the wind that blew was considered a moderate breeze (20-28 kph), compared with 8% in 2019. There were more days during the 2019 flowering period with recorded wind gusts (20 days) than in 2018 (16 days). There is evidence to suggest that a slight variation in wind speed, irrespective of an increase or decrease, has a greater effect on pollen travel than higher wind speed alone (Sauliene & Veriankaite, 2012) and therefore, these wind gusts may play an important role in moving NWR pollen from the pollen donor source.

Distance and Direction of Pollen Travel. Detection of PMF pollen-mediated gene flow via phenotypic observation of the progeny of WMF plots ranged from 0-19% and 0-48% in 2018 and 2019, respectively (Figure 4). In both years, the highest rate of PMF penetrance occurred within the WMF plots bordering or closest to the PMF donor plot(s). This suggests that the highest percentage of pollen-mediated gene flow within NWR occurs within the first 7 m of the pollen donor source. Beyond a 7 m N-S distance, pollen-mediated gene flow from PMF plots to WMF plots ranged from 1.2%-5.3% in 2018 (up to 35 m tested) and 0.0%-2.4% in 2019 (up to 70 m tested) (Table 2). No gene flow was detected past 63 m. However, it is important to note that pollen density would be higher in a larger production field, which could result in higher rates of hybridization. The higher rates of pollen penetrance into the 2018 plots may have been influenced by the amount of donor

pollen available per the experimental design, the higher mean air temperatures, or the lower amounts of precipitation in 2018. While we are unable to confirm using this dataset, we hypothesize that PMF pollen penetrance in 2019 beyond 49 m was caused by contaminant PMF plants that were not identified and rogued in WMF plots prior to pollen shed. These contaminant plants were identified in plots 1934 and 1935. However, further studies need to be conducted to confirm whether or not the 49 m penetrance was due to contamination. Despite the possibility of contamination, the estimates of pollen-mediated gene flow in this study were comparable to those identified in other grass species. In both maize (*Zea mays*) (Messeguer et al., 2006) and common waterhemp *(Amaranthus rudis* Sauer) (Sarangi et al., 2017), evidence of pollen-mediated gene flow via wind was found within the first 50m surrounding pollen donor plants but then dropped off considerably.

To investigate the relationship between the rate of hybridization in WMF plots and distance from the purple pollen donor source among years, we fit a curve to the data from each year and compared the confidence intervals for α and β(Table 3). The model was deemed appropriate for the description of pollen-mediated gene flow in both years by the highly significant parameter estimates and the non-zero confidence intervals. However, this empirical model only includes distance as a predictive factor of pollen-mediated gene flow and does not account for other environmental factors, such as wind. Therefore, we generated an ANOVA using a multiple regression model including distance and wind run. Both variables had a significant effect in both years during the flowering periods. In this regression model, distance had the biggest impact on the rate of hybridization in 2018, while wind run had a bigger impact on hybridization in 2019 (Table 4). The significant

interaction between distance and wind run indicates that distance from the pollen source as well as wind speed and its proportion have an impact on hybridization. These results suggest that as distance from the pollen source increases, wind becomes a more important factor in the rate of hybridization, a phenomenon that has been identified in other species as well (Hanson et al., 2005; Schmidt et al., 2013). While we suggest the possibility that contamination could have led to hybridization at greater distances, it is also possible that wind speed and direction could have led to hybridization from the purple pollen source.

Recombination between Culm, Auricle, and Male Floret Color. In NWR, purple and white color variation also presents in the culm (Figure 3c-d) and auricle (Figure 3e-f), which are hypothesized to be linked to male floret color. To assess the degree of linkage, observations of floret, culm, and auricle color were collected in 2018. Of the 335 analyzed plants, only three plants displayed recombinant phenotypic classes between male floret color, culm color, and auricle color with an estimated 0.9 cM separating culm and auricle color genes (Figure 3g). The limited recombination between these three traits indicates they are tightly linked, which will be helpful for progeny testing in future WMF gene flow studies, as culm and auricle color can be evaluated at earlier stages of development. Genes for purple coloration (anthocyanin accumulation) in various plant parts have been identified in other grain crops including wheat, triticale, and white rice (Dhulappanavar, 1973, 1979; Zeven, 1985).

Conclusions

This study confirmed that the utilization of the male floret color as a means to track pollen-mediated gene flow in NWR is an effective strategy, as long as the WMF seed source is not contaminated with seed harboring the purple male floret color. The contamination in the WMF seed sources highlights the difficulties of controlling pollen travel in cultivated NWR breeding programs. However, pollen-mediated gene flow in this study was limited beyond 7 m from the pollen donor source, providing estimates of pollen-mediated gene flow for establishing new pollen travel mitigation strategies in irrigated paddy settings. The limited range of pollen-mediated gene flow in NWR is further supported by genetic diversity studies that have estimated high genetic differentiation between different lake and river populations of NWR, suggesting limited gene flow among populations (Lu, Waller, & David, 2005; Xu et al., 2015). These results suggest that the possibility of pollen-mediated gene flow between cultivated NWR and natural stands remains low. However, Halsey et al. (2005) indicated that the relative amount of pollen produced from the pollen source is an important factor when evaluating pollen-mediated gene flow in a species. Our study was small in scale in comparison to cultivated paddies as well as natural stands in lakes and river systems. In the future, we suggest a large-scale on-farm study, similar to Cregan (2004), is necessary to effectively determine maximum pollen travel distances in NWR. Additionally, while we identified that air temperature, rainfall, and wind patterns play a role in the distance of pollen travel in NWR, more research is needed to fully understand the effect of weather conditions on pollen travel.

Table 1 Growing Season Weather Data. Mean air temperature (°C) and precipitation (mm) during the experiment in 2018 and 2019 in Grand Rapids, Minnesota, along with corresponding northern wild rice Principal Phenological Stage (PPS). Precipitation values are totaled per month and cumulative precipitation values (in parentheses) give the running total for the entire growing season.

		Mean Air Temperature (°C)			Monthly Precipitation (mm)		
Month	**PPS**[†]	**2018**	**2019**	**20 Year Average**	**2018**	**2019**	**20 Year Average**
May	0	15.2	9.4	11.9	95.50 (95.50)	45.72 (45.72)	87.00
June	1-2	17.8	16.8	17.4	119.38 (214.88)	151.13 (196.85)	115.06
July	2-5	20.4	20.4	20.5	82.55 (297.43)	61.21 (258.06)	84.84
August	5-8	19.1	16.3	18.9	35.05 (332.49)	61.47 (319.53)	79.86
September	7-9	13.7	14.5	14.4	80.26 (412.75)	117.35 (436.88)	81.56

[†]Duquette & Kimball, 2020.

Table 2 Hybridization Rate of White Male Floret Progeny. Effect of north-south (N-S) distance from the pollen donor source on pollen-mediated gene flow from purple male floret (PMF) northern wild rice plots to white male floret (WMF) plots in Grand Rapids, Minnesota during the 2018 and 2019 seasons. Individual plot hybridization data was pooled by range to express the total effect of distance from pollen donor source. Distances that were not able to be measured in 2018 due to spatial limitations are denoted with a hyphen (-).

	Hybridization rate (0.0093SE)	
N-S Distance (m)	**2018**	**2019**
1-7	13.1%	9.2%
8-14	5.3%	0.0%
15-21	1.7%	0.0%
22-28	1.2%	2.4%
29-35	3.9%	0.0%
36-42	-	2.4%
43-49	-	2.4%
50-56	-	2.4%
57-63	-	1.2%
64-70	-	0.0%

Table 3 Hybridization Frequency x Distance Model ANOVA. Analysis of variance and parameter estimates obtained by fitting hybridization frequency to distance from the source population using the model: frequency of hybridization = $\alpha \exp(-\beta \times \text{distance})$.

Year	Source	df	MSE[†]	P>F	Coefficient	Estimate	SE	Confidence Interval	
2018	Model	2	0.0427	<0.0001	α	0.2524	0.0272	0.1952	0.3096
	Error	18	0.0009		β	0.0199	0.0016	0.0166	0.0232
2019	Model	2	0.0540	0.0003	α	0.1256	0.0282	0.0686	0.1826
	Error	41	0.0055		β	0.0076	0.0014	0.0048	0.0104

[†]Mean square error

Table 4 Distance x Wind Run ANOVA. Analysis of variance tests for the effects of distance from purple male floret pollen donor plot(s) and wind run (the amount of wind that traveled through each plot), and their interaction, on the frequency of hybridization in 2018 and 2019.

Year		df	F value	P value
2018	Distance	1	18.916	0.0005
	Wind Run	1	5.690	0.0298
	Distance * Wind Run	1	9.795	0.0065
2019	Distance	1	10.811	0.0021
	Wind Run	1	31.505	0.0002
	Distance * Wind Run	1	7.997	0.0074

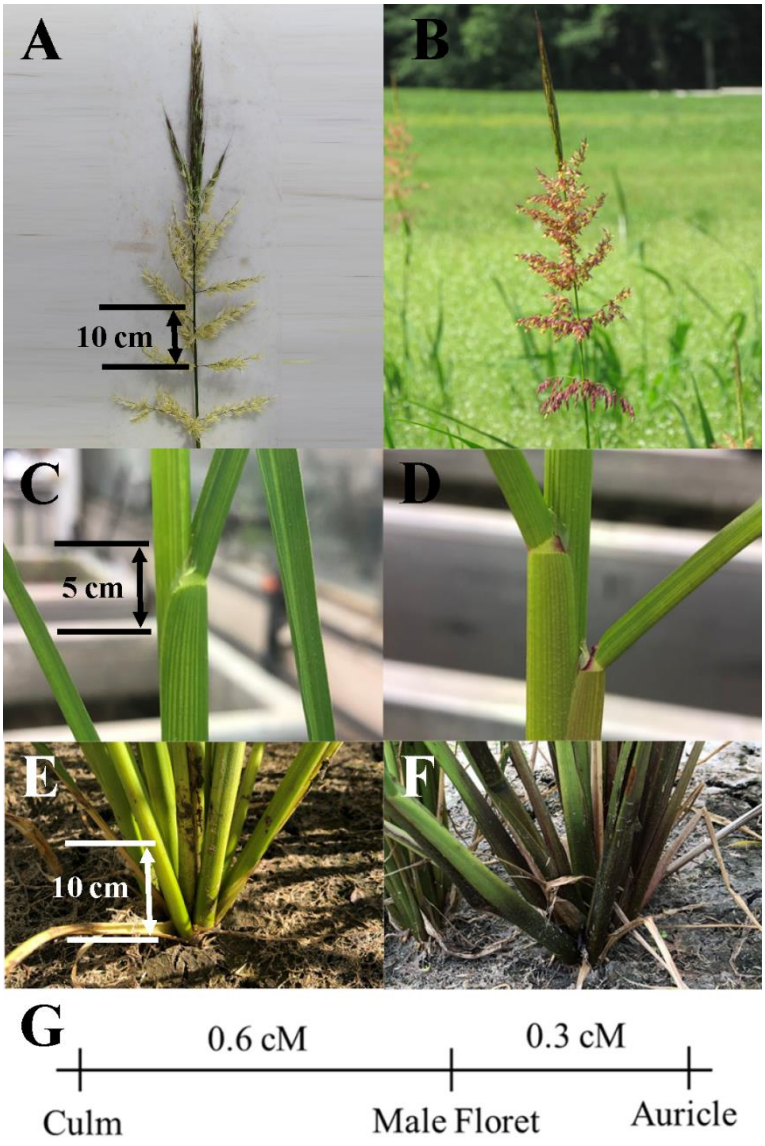

Figure 3 NWR Phenotypes. Phenotypes of the purple male floret (PMF) and white male floret (WMF) colors in northern wild rice (NWR). The left side of the figure represents white phenotypes while the right side represents purple phenotypes. A/B, C/D, and E/F represent the male floret, auricle, and culm, respectively. Approximate measurements are indicated in centimeters for reference, as separate plant part images are not to scale. Finally, G represents the putative recombination map for the genes associated with these traits.

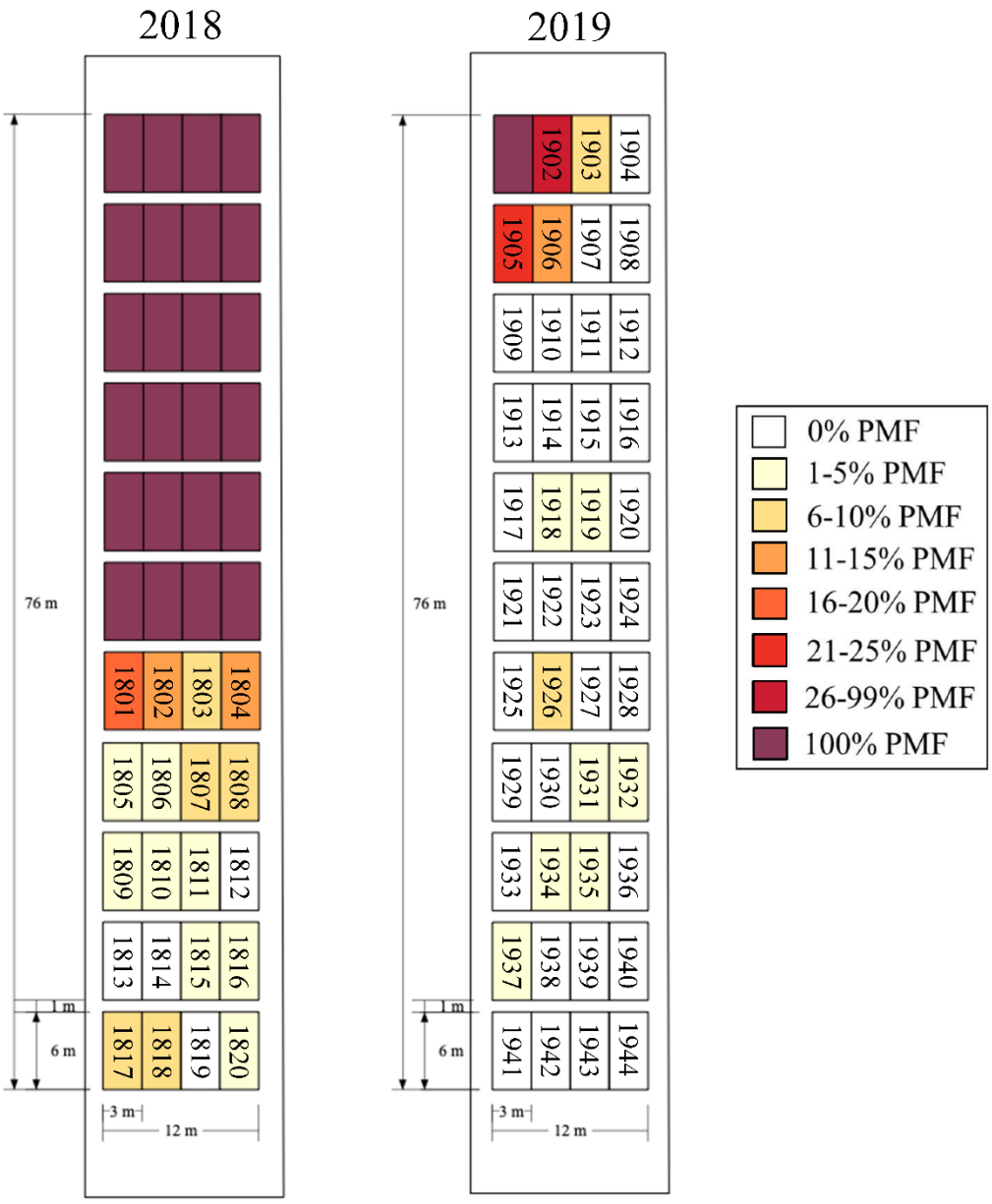

Figure 4 Plot Maps. Plot maps of 2018 and 2019 pollen travel experiments in northern wild rice (NWR) along with hybridization results, which indicate purple male floret (PMF) hybridization rates in the white male floret (WMF) progeny. 100% PMF plots with no plot numbers represent the original PMF pollen donor plots. Numbered plots represent the data collected from the progeny that were originally harvested in those plot locations. White plots represent 100% WMF progeny/0% PMF progeny plots and labeled colored plots represent varying degrees of PMF densities.

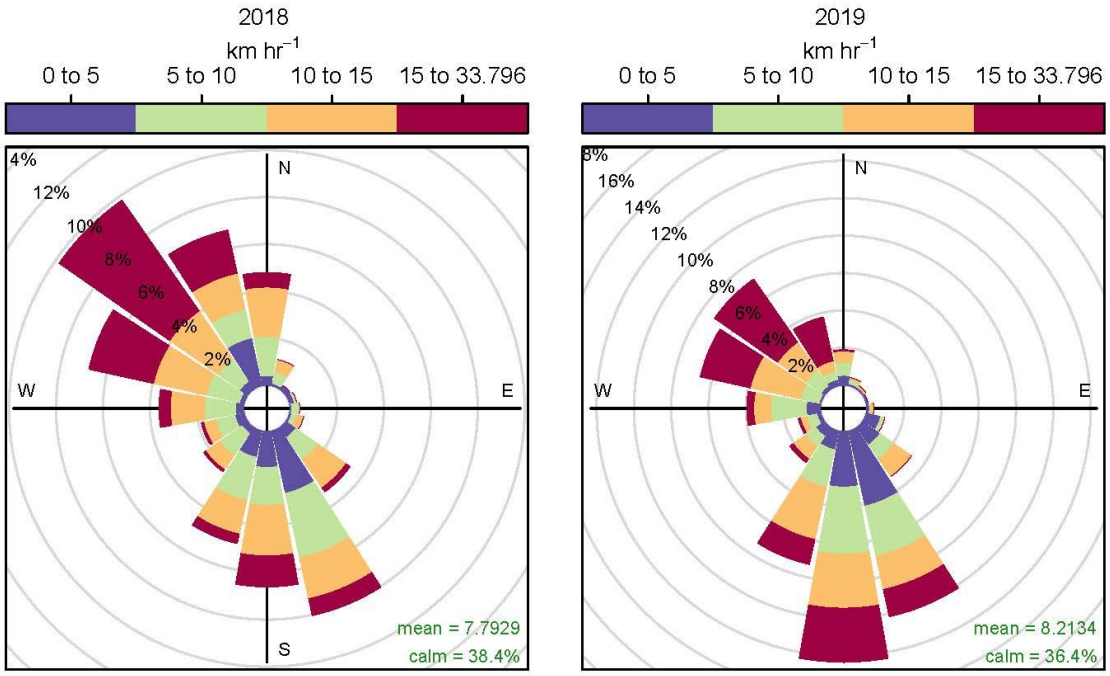

Figure 5 Flowering Period Wind Roses. Wind trends during the 2018 and 2019 flowering periods (July 15- August 15 and July 25-August 25, respectively) at the Itasca County Airport in Grand Rapids, MN. Wind trends show wind blowing from the direction specified. Mean wind speed was 7.79 kph in 2018, and 8.21 kph in 2019. Both years saw similar frequencies of calm air, 38.4% of the time in 2018 and 36.4% of the time in 2019.

Chapter 3 – Spatiotemporal Profiling of Seed-Associated Microbes of an Aquatic, Intermediate Recalcitrant Species, *Zizania palustris* L. and the Impact of Anti-Microbial Seed Treatments

Abstract

Northern wild rice (NWR; *Zizania palustris* var. *interior*) is an ecologically important, annual aquatic species native to North America that is also cultivated in irrigated paddies in Minnesota and California. NWR is desiccation-sensitive and has to be kept in hydrated conditions, during which seed viability declines significantly within the first two years of storage. During this period, microbial growth is rampant and the relationships between these communities and the viability of NWR seed is unclear. In this study, we isolated and genotyped microbes found in 27 NWR seed stocks, collected over a five year period, from three locations, and four genotypes (consisting of three released NWR varieties and one elite breeding line). Results revealed that microbial communities were heavily dependent on seed viability and began shifting after one year of NWR seed storage. Fungi also became more prominent as years in storage increased, suggesting that fungi begin to outcompete bacteria. Spatial analysis between locations and analysis of genotypes revealed a core set of microbes found across locations and genotypes. The genera *Penicillium* and *Pseudomonas* were ubiquitous in hydrated NWR seed storage. We also evaluated the efficacy of four antimicrobial treatments for the reduction of microbial growth in hydrated NWR seed storage. These treatments did not reduce or drastically change microbial growth or seed viability. However, previously undetected microbes were identified after treatments, which suggested a major microbiome shift. Overall, this study identifies

common microbial constituents found in the seed storage of an aquatic, cold-adapted, recalcitrant species and provides a foundation for future studies to evaluate the effect of microbial communities on NWR seed viability during hydrated storage.

Introduction

Northern wild rice (NWR; *Zizania palustris* L.) is an ecologically important, annual, aquatic species native to North America, that is also cultivated in irrigated paddies in Minnesota and California (Biesboer, 2019; Grombacher et al., 1997; E. Oelke & Porter, 2016; Porter, 2019). NWR seed is dormant at harvest, similar to many non-domesticated grasses, and is considered intermediately recalcitrant, or desiccation sensitive, which is fairly unusual for members of the *Poaceae* family (Cardwell, Oelke, & Elliott, 1978; Dickie & Pritchard, 2002; Pammenter & Berjak, 1999; Pence, 1995; Probert & Longley, 1989). Due to its unique seed physiology, NWR is typically stored *ex situ* in the dark and submerged in water at near-freezing temperatures from harvest until planting the following year, to mimic the species' natural aquatic environment (Hayes et al., 1989; Kovach & Bradford, 1992b; Simpson, 1966). In these storage conditions, seed viability and germination rapidly decline, particularly once seed dormancy is broken or around three to six months post-harvest (Berjak & Pammenter, 2008; Grombacher et al., 1997; McGilp et al., 2020; Simpson, 1966). This decline is a major limiting factor in *ex situ* storage such as seed banks, as well as for the maintenance of germplasm in research programs (Raven, Kennedy, Walters, Berjak, & Pammenter, 2013).

One potential cause of the rapid decline of NWR seed viability is the proliferation of microbial growth during submerged storage (Berjak & Pammenter, 2008; Makhathini, 2017; McGilp et al., 2020). Microbial growth, particularly fungal growth, is a main factor in the deterioration of orthodox seed during storage (Christensen & Kaufmann, 1965; El-Dahab, El-Ward, Ibrahim, & Yousof, 2016; Khairnar, Kelhe, & Khairnar, 2011; Kirkpatrick & Bazzaz, 1979). The composition of storage-associated microbes of orthodox seed can be affected by a number of factors including seed moisture content, storage temperature, plant species, and plant-microbe or microbe-microbe interactions (Adam et al., 2018; Cottyn et al.; Nelson, 2004; Truyens, Weyens, Cuypers, & Vangronsveld, 2015; Welty, 1987). However, few studies have evaluated the microbiome of recalcitrant seed, stored in hydrated conditions. Mycock (1990) found that over a period of 21 days *Fusarium* was the predominant seed-associated microbe from seven recalcitrant species (Mycock & Berjak, 1990). More recently, Makhathini (2017) conducted a more comprehensive study of the seed microbiomes of three recalcitrant tree and bush species: *Protorhus longifolia*, *Trichilia dregeana*, and *Garcinia livingstonei,* and the efficacy of seed treatments on the reduction of microbial growth. To the best of our knowledge, no such studies, evaluating the effect of the seed microbiome on seed viability have been conducted using recalcitrant seed from aquatic species.

Due to the interaction between the microbiome and the viability of seed, antimicrobial seed treatments have long been of interest to researchers. As far back as 1651, Samuel Hartlib noted that the brining and subsequent liming of cereal grains reduced the prevalence of smut, resulting in a yield increase in the following growing season (Hartlib,

1651; Smith & Secoy, 1976). Today, the use of antimicrobial seed treatments for the improvement of seed and seedling health is widespread, especially as commercial fungicide seed treatments (FST) have become more prevalent (Lamichhane et al., 2020; White & Hoppin, 2004). In corn, for example, essential oils from mustard seed (*Brassica campestris*), black cumin (*Nigella sativa*), and neem (*Azadirachta indica*) have been used to significantly reduce the growth of major fungal plant pathogens in the genera *Aspergillus, Fusarium, Alternaria,* and *Dreschelera* (Ghafoor & Khan, 1976; Mirza & Qureshi, 1982; Sitara, Niaz, Naseem, & Sultana, 2008). While the use of these essential oils is not common, they were shown to be effective. Sodium and calcium hypochlorite have also been used as antimicrobial seed treatments to reduce the pathogenic load that leads to a decrease in seed quality and an increase in human illness (Sauer, 1986; Schultz & Gabrielson, 1986; Stewart et al., 2001). Chemical fungicides are the most common seed treatments.

Although common in orthodox seed, there are few studies that address the use of antimicrobial treatments for the storage of recalcitrant seed. Most studies conducted with recalcitrant species have utilized surface sterilization treatments for the long-term storage of tissue culture explants, rather than seed (Engelmann, 2012; Farzana et al., 2008; Nower, 2013). A more recent study by Makhatini (2017) showed that some chemical fungicides were effective at reducing the growth of fungal isolates initially collected from three recalcitrant species. The study also showed that one fungicide improved seedling vigor, when used in combination with a surface decontaminant (NaOCl) and seed encapsulation (Makhathini, 2017).

In this study, we calculated the germination and viability of NWR seed stored across 5 years. In addition, the diversity of culturable microbes present in NWR seed storage was evaluated using 16S ribosomal RNA (16S rRNA) and internal transcribed spacer (ITS) sequencing primers. Seed microbes were compared across varieties, locations, and years in storage. Antimicrobial treatments were then applied to NWR seed prior to storage and the effect of treatments was assessed through germination as well as the comparison of microbial genera present prior to and following treatments.

Materials and Methods

Microbial Profiling

Plant Materials. For this experiment 28 seed lots were tested. Seeds from three NWR varieties, Barron, Itasca-C12, and Itasca-C20, and one elite breeding line, FY-C20, were hand harvested across five years, 2014-2018, and from three locations, Clearbrook, Waskish, and Grand Rapids, MN (Table 8). The locations chosen include the University of Minnesota North Central Research and Outreach Center, as well as two of the largest areas for NWR production. After each year's harvest, seed was cleaned of other plant debris, weighed/counted, placed in Ziploc bags with distilled H_2O, and stored at 3 °C in the dark. Seed amount harvested ranged from 1 cup to 1 gallon, and water amount varied based on the volume required to submerge seeds.

Microbial Isolation. Three replications of three individual NWR seeds per genotype and location were placed on both nutrient agar (NA) amended with 50 mg/L of cycloheximide

and on potato dextrose agar (PDA) amended with 300 mg/L of streptomycin. Cycloheximide (Siegel & Sisler, 1963) and streptomycin (Ark, 1954) are fungicidal and bactericidal antibiotics, respectively, and were used to reduce contamination and ensure only bacteria and fungi, respectively, survived on agar plates to reduce overall microbial growth and improve isolation efforts. Seed plates were placed in the dark at 23 °C and evaluated every 24 hours for signs of new microbial growth including changes in color, texture, consistency, or growth pattern of fungal hyphae or bacterial colonies. New microbial growth was subcultured and purified by the transfer of a hyphal tip as previously described in Leyronas, et. al (2012), single spore (Leyronas et al., 2012), or single colony (Stevenson, 2006). To store microbial samples long term, scraped hyphal tissue samples were placed in a 50/50 (v/v) solution of 15% skim milk and 20% glycerol, while swabbed bacterial cell samples were placed in a 15% glycerol stock solution. After sample preparation, all samples were placed in storage at -80 °C.

DNA Extraction. Fungal isolates were re-grown from freezer stock on PDA, amended as described above, and DNA extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). For bacterial isolates, a small amount of bacteria, separate from the samples transferred to long term storage, was placed in 300 µl of TrisEDTA (TE) buffer to preserve the cells before DNA extractions. Bacterial DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). The concentration of all microbial DNA was quantified using a NanoDrop 2000 microvolume spectrophotometer (Thermo Fischer Scientific, Waltham, MA).

PCR and Sequencing. Fungal DNA from the internal transcribed spacer (ITS) was amplified using the ITS1F (Gardes & Bruns, 1993) and ITS4 (Bruns, Lee, & Taylor, 1990) universal primers (Table 12). For bacterial DNA, 16S ribosomal RNA (16S rRNA) was amplified using the 27F (Weisburg, Barns, Pelletier, & Lane, 1991) and 1492R (Turner, Pryerb, Vivian, & Palmera, 1999) primers (Table 12). Each reaction contained 50 ng template DNA, 12.5 µL GoTaq Green Master Mix (Promega Corporation #M7123, Madison, WI), 10 µM forward and reverse primers, and nuclease free water. Amplifications were run on a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). Thermocycler conditions for ITS primers were as follows: a 5 min denaturation step at 95 °C; 34 cycles of 95 °C for 30s; 55 °C for 30s; 72 °C for 60s; and a final extension step of 72 °C for 10 min. Conditions for 16S rRNA primers included the following: a 5 min at 95 °C; 30 cycles of 95 °C for 30s; 58 °C for 30s; 72 °C for 50s; and a 10 min extension step at 72 °C. Amplicons were sent to Molecular Cloning Laboratories in San Francisco, California for PCR clean up using ExoSAP-IT™ enzymatic PCR clean-up (Thermo Fischer Scientific, Waltham, MA) and Sanger sequencing on a ABI 3730X DNA analyzer (Thermo Fischer Scientific, Waltham, MA).

Germination and Tetrazolium Testing. Seed viability was estimated using both germination and tetrazolium testing. For germination tests, ~20-30 seeds were rinsed in distilled H_2O, placed in a new Ziploc bag, and positioned in a growth chamber for three weeks with 15 hours of light at 18 °C and 9 hours of dark at 9 °C. Germination was defined as coleoptile

emergence of 1 cm. Tetrazolium tests were conducted according to the Association of Official Seed Analysts 2000 - Tetrazolium testing handbook protocol (Association of Official Seed Analysts. Tetrazolium Testing Committee, 1970). Seeds that did not germinate during germination testing were bisected down the long axis of the seed to expose the embryo, and suspended in a 0.1% solution of tetrazolium blue chloride (Sigma-Aldrich #88190, St. Louis, MO) for 2 hours. Seeds were then rinsed and observed for uptake of the stain by the embryo, which indicated metabolically active tissue that could potentially germinate into a viable plant.

Data Analysis. To compare germination between years, genotypes, and locations, averages and standard errors were calculated for each year, genotype, and location using R Studio version 1.2.5001 (R Studio Team, 2021). Tetrazolium results were treated as a binary; as long as at least one seed per sample stained, it was considered to be a viable seed set. Geneious Prime was used to trim 16S and ITS DNA sequences (Geneious Prime 2019.2.3, https://www.geneious.com). Sequences were run through the NCBI Nucleotide Basic Local Alignment Search Tool (BLASTn) and identified to the genus level, by selecting the top ranked match. Microbial genera were then compared across years, genotypes, and locations, as well as by viable and non-viable seed samples. Figures were also created in R Studio. All packages used in R Studio can be found in Table 10.

Anti-Microbial Seed Treatments

Plant Materials. To evaluate the influence of microbial growth on seed quality in NWR during storage, chemical treatments were utilized in an attempt to reduce microbial growth. Three varieties, Barron, Itasca-C12, and Itasca-C20, and one elite breeding line, FY-C20, from two different locations, Grand Rapids and Clearbrook, MN, tested in 2018 were utilized for this experiment. Before any seed treatments took place, the microbes associated with these genotypes were isolated and reported in the Microbial Profiling of NWR Seed section.

Antimicrobial Seed Treatments. Two antimicrobial agents, 10% bleach and 10% hydrogen peroxide, were evaluated for two different rinse lengths, one minute and five minutes. In order to improve the contact angle of the bleach solution, 100 µl of Tween 20, a nonionic surfactant, was added to produce a 10% bleach, 0.01% Tween solution.

Three replications of 100 seeds of each genotype by location were placed in 4 oz., 5.08 cm diameter, polystyrene jars. Prior to antimicrobial treatment, seeds were rinsed in 70% ethanol for one minute to aid in the breakdown of the seed's waxy coating, and then triple rinsed with deionized water. Seeds were then treated with either the 10% bleach solution or 10% hydrogen peroxide for either one minute or five minutes, and then triple rinsed with deionized water. For storage, seeds were covered in deionized water and placed in the dark at 3 °C for 27 weeks until germination testing. For the control treatment, seeds were pretreated with 70% ethanol, triple rinsed with deionized water, covered with

deionized water, and placed in storage. There were 120 jars in total (4 genotypes x 2 locations x 5 treatments x 3 replications).

Post-Treatment Microbial Profiling. The microbes that survived antimicrobial treatments were isolated 32 weeks after treatment. Three replications of three seeds per treatment by location by genotype combination were placed on both PDA and NA. Methods for microbial isolation followed the pre-treatment isolation methods described above.

Germination Testing. Viability of treated seed via germination testing was analyzed 27 weeks after treatment. Forty seeds from each treatment jar were placed in a ULINE 2mil 3”x5” plastic sample bag and filled with 10 ml deionized water. Bags were placed under a benchtop grow light programmed for 16 hour days. Ambient temperature was approximately 22 °C. Seeds were monitored for 14 days. A seedling was officially deemed germinated when the coleoptile showed signs of greening and the length of the coleoptile surpassed that of an un-germinated, dissected seed (approx. 1 cm). Germination was also estimated the following year (~90 weeks after treatment) using the remaining seed that had not previously germinated in storage or seed that had its seed coat rupture prematurely but the coleoptile did not elongate.

Data Analysis. Analysis of 16S rRNA and ITS DNA sequences were analyzed with Geneious Prime. Sequences were trimmed and aligned to known fungal and bacterial sequences using the Basic Local Alignment Search Tool (BLASTn). Analysis of variance

was generated using R (R Core Team, 2020) with fixed effects of location, replication, genotype, and treatment. Using the LSD test function with the Bonferroni adjustment, the least significant differences were calculated for location, genotype, and treatment.

Results and Discussion

Microbial Profiling of NWR Seed. Host-associated core microbiomes are complex ecosystems that can improve our understanding of important biological functions within host species. Research in this area has been developing rapidly with a large emphasis on agricultural crops, including *Triticum*, *Zea*, and *Oryzae* (Eyre, Wang, Oh, & Dean, 2019; Johnston-Monje & Raizada, 2011; Kuźniar et al., 2020). In this study, we sought to characterize the presence and changes in microbial communities within NWR seed storage over time in storage, across locations, and between genotypes. Samples were collected from four genotypes, collected at three cultivated NWR sites, and over a five-year period. Across all genotypes, locations, and years, 27 total microbial taxa were isolated and identified to the genus level, including 14 fungi and 13 bacteria, which were spread across five divisions and 11 classes. We found 13 genera belonging to seven different orders in Ascomycota (48.1%), one genus in Mucoromycota (3.7%), two genera from one order in Bacteroidetes (7.4%), two genera from one order in Firmicutes (7.4%), and nine genera spread across five different orders in Proteobacteria (33.3%) (Table 5). Across samples, the most prevalent Ascomycetes included *Penicillium* (26 samples), *Geotrichum* (15), and *Candida* (9). Only two genera, *Pseudomonas* and *Penicillium*, were found across years,

locations, and genotypes (Table 8). Both genera are considered ubiquitous generalists and are commonly isolated from a variety of substrates and locations (Chakravarty & Anderson, 2015; Visagie et al., 2014). An additional 30 isolates were not identified due to difficulty during DNA extractions or anomalies during sequencing, which indicates that future studies are needed and may benefit from high-throughput sequencing methods, which would additionally provide information about non-culturable microbes.

Seed Viability Impacts the NWR Seed Microbiome. The seeds of desiccation-sensitive, recalcitrant species, such as NWR, can only survive in *ex situ* storage for a limited time (Raven et al., 2013; Tweddle, Dickie, Baskin, & Baskin, 2003). For some species of tropical forest trees, such as *Inga vera* and *Syzygium cumini*, it is only days to months (Nagendra, Tamilarasan, Shobha, Ragupathi, & Renganayaki, 2019; Parisi, Biagi, Medina, & Barbedo, 2016; Vázquez-Yanes & Orozco-Segovia, 1993), and for others, like NWR, seed can survive and maintain vigor for approximately one year (Berjak & Pammenter, 2008; Grombacher et al., 1997; Simpson, 1966). During that time, microbial growth can become abundant (Berjak & Pammenter, 2008). While the presence and consequence of microbes found in orthodox seed storage have been well studied, we know little about the presence and impact of microbial communities in hydrated storage (Calistru, McLean, Pammenter, & Berjak, 2000). In this study, we found that seed viability played a major role in the microbial constituents of submerged NWR seed storage. (Table 5; Figure 6a). Due to the short duration of NWR seed viability within our current storage system, we anticipated that seed stored for more than two years would, at best, display low

germination. Germination and tetrazolium testing confirmed that all but one sample from 2014-2016 seed stocks, representing three to five years in storage, were non-viable (Figure 6a; Table 9). The average germination of 2017 seed, which had been stored for two years, was 53.1 % and 33.8 % in 2018 (Figure 6a). The average number of microbial genera identified from 2014-2016 and 2017-2018 seed stocks was six and 14, respectively (Figure 6d), demonstrating that the microbial communities from viable seed were more diverse than from non-viable seed. In addition to harboring a more diverse microbiome, 2018 seed also contained the most unique genera (defined as total number of genera identified rather than number of samples per genus), by a large margin (13 unique genera in 2018, $\leq$3 in remaining years) (Figure 7a). The microbial genera found in 2017 seed were interesting in that they overlapped with genera found on both viable and non-viable seed from the other years (Figure 7a; Table 8). Therefore, it seems that the microbial community found in 2017 seed, after one year of storage, may represent a transitional phase between viable and non-viable seed. We also identified a shift in the ratio of fungal and bacterial genera, with a roughly 1:1 ratio in newer seed, but a skew towards more fungal genera in older seed (Figure 6d). These results indicate that microbial diversity in NWR hydrated storage declines with the age, and associated viability, of the seed, and that over time, fungi outcompete bacteria. This association between the decline of seed viability and an increase in fungal activity has also been identified in other recalcitrant species (Calistru et al., 2000). The decrease in microbial diversity, following the loss of seed viability, could also be related to a decline in nutrient availability as well as the potential accumulation of

antimicrobial substances secreted by the dead organ tissue surrounding the embryo, or by other microbes (Godwin, Raviv, & Grafi, 2017; Raviv, Godwin, Granot, & Grafi, 2018).

Temporal Analysis of Microbial Community Structures in NWR Storage. Storage time appears to have a dramatic effect on the composition and diversity of microbial growth on NWR seed. In this study, we assessed the microbial communities from NWR seed stocks that were in storage for one to five years. Microbes were identified from all seed stocks, regardless of viability, which allowed us to investigate putative shifts in microbial communities throughout storage times. Given that storage time is confounded with year effect, we were unable to get the most accurate analysis of the effect of seed age on culturable microbial communities. There were nine microbial genera found in both viable and non-viable seed: the fungal genera *Penicillium*, *Talaromyces*, *Pseudeurotium*, *Pichia*, *Candida*, *Geotrichum*, and *Cosmospora* and the bacterial genera *Bacillus* and *Pseudomonas* (Table 5). Many of these genera are ubiquitous, including *Penicillium*, *Pseudomonas*¸ and *Bacillus* and have been associated with a number of host microbiomes (Adam et al., 2018; Eyre et al., 2019; Links et al., 2014; Visagie et al., 2014). Although found in both viable and non-viable seed, *Talaromyces*, *Pichia,* and *Geotrichum* species have been associated with the break-down of seed coats, dehiscence, and decomposition (Kim et al., 2017; Sfakianakis et al., 2007; Telli-Okur & Eken-Saraçoğlu, 2008). *Flavobacterium*, is known to efficiently degrade biopolymers and dissolved organic matter, which becomes available as plants decompose (Bernardet & Bowman, 2006; Hur et al., 2011).

There was a larger diversity and number of microbes unique to seed from 2017 and 2018, representing the shortest storage lengths, than other years (Figure 6d). A total of 16 genera were unique to either 2017 or 2018 seed, including seven fungal and nine bacterial genera, which were spread out across four divisions and eight classes (Table 5; Table 8). Of these, only three fungal genera, *Candida*, *Galactomyces* and *Pseudeurotium*, were unique to 2017 seed. There were four genera found in both 2017 and 2018 seed: *Pseudomonas* and *Penicillium*, which are ubiquitous, as well as *Chryseobacterium* and *Mucor*. Of the unique microbes from 2017 and 2018, some putative functions were identified. The promotion of plant growth has been found for species of *Achromobacter* (Corsini et al., 2018; Jiménez-Vázquez et al., 2020), *Acinetobacter* (Shi, Lou, & Li, 2011), *Chryseobacterium* (Veer & Goel, 2015), *Delftia* (Suchan et al., 2020), and *Stenotrophomonas* (Schmidt, Alavi, Cardinale, Müller, & Berg, 2012). A few of these unique microbes have also been assessed for their production of antimicrobial substances or use as biocontrol agents including species of *Delftia* (Prasannakumar, Gowtham, Hariprasad, Shivaprasad, & Niranjana, 2015), *Enterobacter* (Gong et al., 2019), *Serratia* (Johnson, Pearson, & Jackson, 2001), and *Sphingomonas* (Wachowska, Irzykowski, Jędryczka, Stasiulewicz-Paluch, & Głowacka, 2013). Several of the fungal genera identified here, including species of *Alternaria* (Casa et al., 2012; Gambacorta et al., 2018), *Cladosporium* (Schenck & Stotzky, 1975), *Epicoccum* (Anžlovar, Likar, & Koce, 2017; Schenck & Stotzky, 1975), *Fusarium* (Gambacorta et al., 2018; Mycock & Berjak, 1990), and *Penicillium* (Gambacorta et al., 2018; Mycock & Berjak, 1990) commonly accumulate during grain seed storage, with some species ultimately leading to seed deterioration or the

production of mycotoxins (Magan, Sanchis, & Aldred, 2004). Still others are pathogenic to a variety of hosts including species of *Alternaria* (Babadoost, 2011; Maude & Humpherson-Jones, 1980), *Cladosporium* (Kirk, 1984; Nam, Park, Kim, Kim, & Kim, 2015), *Epicoccum* (Stokholm et al., 2016), *Ewingella* (González, Gea, Navarro, & Fernández, 2012), *Fusarium* (Babadoost, 2011), and *Galactomyces* (Song et al., 2020). Ultimately, due to the complexity of host-microbe interactions, it is difficult to draw broad conclusions about the function of these microbes in NWR seed storage (Hardoim et al., 2015). Further testing of microbial interactions in NWR is needed to clarify the true function(s) of these microbes in this unique ecosystem.

Spatial Analysis: Core Microbes across Locations and Genotypes. Environmental factors, such as local climate, soil composition, nutrient availability, and water, which vary based on growing region or even field location, all can affect the microbial community composition of plant species (Buyer et al., 1999; Hacquard, 2016). Within this study, we evaluated three different sites across northern Minnesota common to NWR cultivation and identified differences in the number of genera between locations (Figure 7b). Five genera were common to all locations, possibly indicating that these microbes are found within, rather than on the surface of seed, and could be part of a core NWR microbiome. These included *Bacillus*, *Candida*, *Geotrichum*, *Pichia*, and *Pseudomonas*. Many of these microbes have been associated with the deterioration or decomposition of various tissues in other species, such as *Orchidea* (Sfakianakis et al., 2007), several fruits species, including tomato (Nishikawa, Kobayashi, Shirata, Chibana, & Natsuaki, 2006; Talibi et

al., 2012), and sunflower (Telli-Okur & Eken-Saraçoğlu, 2008). The low number of unique microbes identified in Waskish could be due to the lower number of samples tested (n=4, compared with Clearbrook, n=10, and Grand Rapids, n=14) (Figure 6e). Each location had their own management practices for cultivation as well as unique weather conditions, which may have influenced the type of microbes identified at these locations. While location-specific differences in microbial communities are important to understand, they are just one piece of the complex interaction between environmental conditions and host species, and their influence on microbial communities.

Plant microbial communities are vertically transmitted from generation to generation, which can lead to genotype-specific differences in microbial communities (Adam et al., 2018; Johnston-Monje, Lundberg, Lazarovits, Reis, & Raizada, 2016; Nelson, 2004; Shade, Jacques, & Barret, 2017). However, it is important to note that vertical transmission was never explicitly tested in this experiment while exploring genotype-specific microbiome differences. We evaluated four different genotypes in this study to identify potential differences between genotypes of NWR. Similar numbers of microbial genera were found in Barron, FY-C20, and Itasca-C12 samples (14, 16, and 14, respectively) (Figure 6f). By comparison, only nine genera were isolated from Itasca-C20 samples. The lower diversity of microbes in Itasca-C20 could be due to the low sample number from this variety that was used in this study (n=2). *Mucor*, *Penicillium*, and *Pseudomonas* were found in all genotypes. *Acinetobacter*, *Bacillus*, *Candida*, *Chryseobacterium*, *Cosmospora*, *Geotrichum*, and *Pichia* were found in three out of the four genotypes. We did not identify any consistency, however, in microbial communities

based on genotype when exploring the interaction between genotype, location, and year. The microbial structure, again, seems to be largely dictated by viability of seed and more testing on viable seed, in general, is needed to confirm these findings.

Antimicrobial Seed Treatments Study Reveals Microbial Communities in NWR Can Shift Rapidly. This study sought to identify the effect of seed treatments on the germination and diversity of microbes present on NWR seed following storage. For this experiment, seed data from the 2018 samples, described in the previous section, was used as a pre-treatment comparison to identify differences between seed prior to treatment and post-treated seed. Following treatment and approximately eight months of storage, seed germination and microbial growth were assessed for each treatment, genotype, and location. Differences in germination between locations, genotypes, and the interactions of treatment/location and location/genotype were found in this study (Table 6). Although the ANOVA indicated that there was a significant interaction between treatment and location, the only differences found using a post-hoc Tukey's test were due primarily to differences by location. This implies that any differences between treatments were quite small.

The microbiome from seed prior to treatment and prior to the storage period differed (pre-treatment seed) from that of the control and treated seed, after an eight month storage period (Table 7). Seven microbial genera were found only in the pre-treatment seed: *Achromobacter*, *Arthrinium*, *Delftia*, *Enterobacter*, *Epiccocum*, *Ewingella*, and *Penicillium*. Because these genera were not found in control seed, their loss potentially represents a natural shift in microbial diversity over approximately eight months in

submerged storage. Additionally, there were 13 microbial genera found in the control but not pre-treatment seed. Ultimately, pre-treatment and control seed shared only 5 of 31 identified microbial genera: *Chryseobacterium*, *Fusarium*, *Pseudomonas*, *Serratia*, and *Sphingomonas* (Table 7). This dramatic shift in the microbial composition, between pre-treatment and control seed, may reflect the change from a dry environment to submerged storage, which decreases the availability of oxygen, changes in nutrient availability, or the effect of seed germination following the natural dormancy period, and the associated exudates (Nelson, 2004; Shade et al., 2017; Truyens et al., 2015). However, to the best of our knowledge, there are no published studies comparing the microbiome of recalcitrant seed, before and following submerged storage that would aid in the development of more concrete conclusions about the observed phenomenon.

Antimicrobial Seed Treatments Did Not Have A Strong Effect on Germination or Microbial Growth. The seed treatments used in this study did not appear to strongly influence the germination or the decline of microbial growth on seed, when compared to the control. There were no statistical differences in the germination of control versus treated seed or between seed treatments (Table 6). Likewise, it appears that the loss of microbial genera, compared to control seed, cannot be linked to any of the tested treatments, as there were no microbial genera shared by the control and pre-treatment seed that were not also found in the treated seed. However, 13 microbial genera were found in treated seed but not in the control, suggesting that treatments allowed for the growth of otherwise undetected microbes. Surface sterilization via antimicrobial treatments may effectively control surface

microbes, but may also create favorable conditions for internal microbes to occupy external seed tissues (Blanchard & Hanlin, 1973; Mohamed, 2017).

While similar numbers of microbial genera were present across all treatments (16-21), only seven of these genera were shared, including the control: *Chryseobacterium*, *Fusarium*, *Pseudomonas*, *Rhodotorula*, *Serratia*, *Sphingomonas*, and *Xanthomonas*. These microbes represent a core microbiome that was unaffected by the seed treatments, which matches closely with the core microbes identified between pre-treatment and control seed. *Pseudomonas*, *Sphingomonas*, and *Fusarium* are genera commonly found in the core microbiome of crop species including *Cucurbita pepo*, *Brassica napus*, *Oryza sativa*, *Triticum aestivum*, and *Lens culinaris* (Adam et al., 2018; Berg et al., 2020; Eyre et al., 2019; Morales Moreira, Helgason, & Germida, 2020). It has been noted previously that *Pseudomonas* and *Fusarium* are not always eliminated from seed following treatment with sodium hypochlorite (Cuero, Smith, & Lacey, 1986). However, other seed treatments have been efficacious in the elimination or reduction of fungi commonly associated with recalcitrant seed, including *Penicillium* spp., *Cladosporium* spp., and *Fusarium* spp (Francoso & Barbedo, 2016; Oliveira, Oliveira, Parisi, & Barbedo, 2011; Parisi et al., 2016). This suggests that different seed treatments than those tested here may be more effective in the reduction of microbial growth during submerged NWR seed storage.

The Effect of Environment and Genotype on the Microbial Constituents Post-Antimicrobial Treatments. Location appeared to influence the germination and microbial diversity of seed in this study. Averaged across treatments, Clearbrook seed had significantly higher

germination than Grand Rapids (Figure 8a). This may be related to the health of the parental plants, which were grown under differing environmental conditions and management practices. Control seed was used to identify the effect of location on the microbial diversity of seed. Only 3 of 17 microbial genera were found in seed from both locations (Table 11). This implies that the parental environment may have influenced the seed microbiomes. Previous research has found differences in microbial diversity of crops including *Phaseolus vulgaris*, *Triticum aestivum*, *Brassica napus*, and *Lens culinaris* seed between locations, owing both to differences in the inherent environment as well as the management of the land (Barret et al., 2015; Hardoim et al., 2015; Klaedtke et al., 2016; Morales Moreira et al., 2020).

Genotype affected both the germination and the microbial community present on seed. When averaged across treatments, FY-C20 had the highest germination, followed by Barron and Itasca-C12, and finally, Itasca-C20 (Figure 8b). Although differences were found between genotypes, it should be noted that the germination of NWR varieties often fluctuates between years. Therefore, it is possible that across years, these differences would be decreased or eliminated. The control seed lots were used to compare the natural progression of storage microbiota over time, between the four NWR genotypes. Only 2 of 17 genera were shared between all genotypes: *Pseudomonas* and *Sphingomonas*, with an additional four genera shared between at least two of the genotypes: *Chryseobacterium*, *Fusarium*, *Pichia*, and *Serratia*. Together, these shared genera make up the core microbiome as described above. This indicates that the core microbiome remains mostly unchanged between genotypes but that genotypes do harbor differing microbes. It is not

uncommon for genotype to play a role in the microbiome of seed, in some cases due to the transmission of endophytes by the parental plant, as has been seen in other plant species (Morales Moreira et al., 2020; Shade et al., 2017; Tannenbaum et al., 2020).

Conclusions

In this study, we investigated temporal and spatial changes of the microbial communities present within NWR hydrated storage. Microbial constituents were largely dependent on the viability of NWR seed, with diversity declining and shifting to fungal-dominated communities over time. With the molecular tools utilized in this study it was not possible to identify microbes to the species level, therefore it is unclear whether isolates may have been pathogenic. However, *Pseudomonas syringae* and multiple species in the genus *Fusarium* are known to cause bacterial leaf streak and Fusarium head blight of wild rice, respectively (Bowden & Percich, 1983; Nyvall, Percich, & Mirocha, 1999). Therefore, it may be useful to further characterize the *Fusarium* and *Pseudomonas* isolates that were found in this study. The antimicrobial treatments analyzed were not effective in reducing the prevalence of microbial growth but support the hypothesis that microbes within the seed endosperm and embryo may shift from an endophytic to epiphytic lifestyle when a suitable environment becomes available. Further testing will clarify the type and timing of more effective treatments. Storage conditions for aquatic, recalcitrant species continue to make the control of microbial growth difficult, and improved storage methods may be necessary to effectively control microbial growth in storage.

Table 5 Identified Microbes. List of microbes identified on northern wild rice (NWR; *Z. palustris*) seed stored submerged in water at 3 °C for 1-4 years. Genera were identified via ITS DNA and 16S rRNA sequencing. *Seed Samples: The number of seed samples used in this study in which microbial genera were found. **Viable/Non-Viable: V-the microbial genus was found only in viable seed samples, N- the microbial genus was only found in non-viable seed samples, B- the microbial genus was found in both viable and non-viable seed samples.

Kingdom	Division	Class	Order	Family	Genus	Seed Samples*	Viable/Non-Viable Seed**
Fungi	Ascomycota	Dothideomycetes					
			Capnodiales				
				Davidiellaceae			
					Cladosporium	2	V
			Pleosporales				
				Didymellaceae			
					Epicoccum	1	V
				Pleosporaceae			
					Alternaria	1	V
		Eurotiomycetes					
			Eurotiales				
				Trichocomaceae			
					Penicillium	12	B
					Talaromyces	2	B
		Leotiomycetes					
			Thelebolales				
				Pseudeurotiaceae			
					Pseudeurotium	3	B
		Saccharomycetes					
			Saccharomycetales				
				Saccharomycetaceae			
					Pichia	6	B
					Candida	9	B
				Dipodascaceae			
					Geotrichum	15	B
					Galactomyces	3	V
		Sordariomycetes					
			Hypocreales				
				Nectriaceae			
					Cosmospora	4	B
					Fusarium	5	V
			Xyriales				
				Apiosporaceae			
					Arthrinium	1	V
	Mucoromycota	Mucoromycetes					
			Mucorales				
				Mucoraceae			
					Mucor	12	V

Kingdom	Division	Class	Order	Family	Genus	Seed Samples*	Viable/Non-Viable Seed**
Bacteria	Bacteroidetes	Flavobacteriia					
			Flavobacteriales				
				Flavobacteriaceae			
					Flavobacterium	1	N
					Chryseobacterium	3	V
	Firmicutes	Bacilli					
			Bacillales				
				Bacillaceae			
					Bacillus	3	B
					Lysinibacillus	1	N
	Proteobacteria	Alphaproteobacteria					
			Sphingomonadales				
				Sphingomonadaceae			
					Sphingomonas	1	V
		Betaproteobacteria					
			Burkholderiales				
				Alcaligenaceae			
					Achromobacter	1	V
				Comamonadaceae			
					Delftia	1	V
		Gammaproteobacteria					
			Enterobacterales				
				Enterobacteriaceae			
					Enterobacter	1	V
				Yersiniaceae			
					Ewingella	1	V
					Serratia	1	V
			Pseudomonadales				
				Pseudomonadaceae			
					Pseudomonas	26	B
				Moraxellaceae			
					Acinetobacter	3	V
			Xanthomonadales				
				Xanthomonadaceae			
					Stenotrophomonas	1	V

Table 6 Treatment Germination Rate ANOVA. Analysis of Variance (ANOVA) for the assessment of the influence of five antimicrobial seed treatments on the germinability of four northern wild rice (NWR; *Z. palustris*) genotypes from two locations in 2018.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Reps	2	459.3562794	229.6781397	2.385230918	0.098752874
Treatments	4	322.0846374	80.52115935	0.836220457	0.506286637
Locations	1	16635.10289	16635.10289	172.7572411	1.79E-21
Genotypes	3	15219.83506	5073.278353	52.6865134	1.03E-18
Treatment x Location	4	1056.863304	264.2158259	2.743908314	0.034226132
Treatment x Genotype	12	1245.621246	103.8017705	1.077991979	0.390059951
Location x Genotype	3	5974.550167	1991.516722	20.68210439	5.88E-10
Trt. x Loc. x Genotype	12	1762.130137	146.8441781	1.524991774	0.13311019
Residuals	78	7510.759129	96.2917837	#N/A	#N/A

Table 7 Identified Microbes Post-Treatment. List of microbes identified post-application of five different antimicrobial seed treatments on northern wild rice (NWR; *Z. palustris*) seed. Seed was collected in 2018 and stored submerged in water at 3 °C for ~8 months post-treatment before testing. Y's denote that the microbial genus was identified on seed with that treatment, while N's denote that genus was not identified on seed samples with that treatment.

Kingdom	Phylum	Class	Order	Family	Genus	Pre-treatment	Control	Bleach 1 minute	Bleach 5 minutes	H2O2 1 minute	H2O2 5 minutes
Fungi	Ascomycota	Dothideomycetes									
			Capnodiales								
				Davidiellaceae							
					Cladosporium	Y	N	Y	N	N	N
			Pleosporales								
				Didymellaceae							
					Epicoccum	Y	N	N	N	N	N
				Pleosporaceae							
					Alternaria	Y	N	Y	Y	N	Y
		Eurotiomycetes									
			Eurotiales								
				Trichocomaceae							
					Penicillium	Y	N	N	N	N	N
		Leotiomycetes									
			Helotiales								
				Ploettnerulaceae							
					Cadophora	N	N	Y	N	N	N
		Saccharomycetes									
			Saccharomycetales								
				Metschnikowiaceae							
					Metschnikowia	N	Y	N	N	N	Y
				Saccharomycetaceae							
					Candida	N	N	Y	Y	N	Y
					Kluyveromyces	N	Y	N	N	Y	N
					Pichia	N	Y	N	N	N	N

				Pichiaceae							
					Nakazawaea	N	Y	N	N	N	N
		Sordariomycetes									
			Coniochaetales								
				Coniochaetaceae							
					Coniochaeta	N	Y	Y	N	N	N
			Hypocreales								
				Nectriaceae							
					Cosmpospora	N	N	N	N	N	Y
					Fusarium	Y	Y	Y	Y	Y	Y
				Sarocladiaceae							
					Sarocladium	N	Y	N	N	N	Y
			Xyriales								
				Apiosporaceae							
					Arthrinium	Y	N	N	N	N	N
	Basidiomycota	Cystobasidiomycetes									
			Cystobasidiales								
				Cystobasidiales incertae sedis							
					Occultifur	N	N	N	Y	N	N
		Microbotryomycetes									
			Sporidiobolales								
				Sporidiobolaceae							
					Rhodotorula	N	Y	Y	Y	Y	Y
		Tremellomycetes									
			Tremellales								
				Cystofilobasidiaceae							
					Cystofilobasidium	N	Y	N	N	N	N
				Phaeotremellaceae							
					Phaeotremella	N	Y	N	N	N	N
				Tremellaceae							
					Papiliotrema	N	Y	N	Y	N	N

Kingdom	Phylum	Class	Order	Family	Genus	Pre-treatment	Control	Bleach 1 minute	Bleach 5 minutes	H2O2 1 minute	H2O2 5 minutes
Fungi	Mucoromycota	Mucoromycetes									
			Mucorales								
				Mucoraceae							
					Mucor	Y	N	N	Y	N	Y
Bacteria	Actinobacteria	Actinobacteria									
			Actinomycetales								
				Nocardiaceae							
					Rhodococcus	N	N	N	N	Y	N
	Bacteroidetes	Flavobacteriia									
			Flavobacteriales								
				Flavobacteriaceae							
					Flavobacterium	N	N	Y	Y	Y	N
					Chryseobacterium	Y	Y	Y	Y	Y	Y
		Sphingobacteriia									
			Sphingobacteriales								
				Sphingobacteriaceae							
					Mucilaginibacter	N	Y	N	N	Y	Y
					Pedobacter	N	N	Y	N	Y	N
	Proteobacteria	Alphaproteobacteria									
			Sphingomonadales								
				Sphingomonadaceae							
					Novosphingobium	N	N	Y	Y	Y	Y
					Sphingobacterium	N	N	Y	Y	Y	Y
					Sphingomonas	Y	Y	Y	Y	Y	Y
		Betaproteobacteria									
			Burkholderiales								
				Comamonadaceae							
					Comamonas	N	Y	N	N	Y	N
					Delftia	Y	N	N	N	N	N
				Oxalobacteraceae							
					Janthinobacterium	Y	N	N	Y	N	Y

Kingdom	Phylum	Class	Order	Family	Genus	Pre-treatment	Control	Bleach 1 minute	Bleach 5 minutes	H2O2 1 minute	H2O2 5 minutes
				Alcaligenaceae							
					Achromobacter	Y	N	N	N	N	N
		Gammaproteobacteria									
			Enterobacterales								
				Yersiniaceae							
					Ewingella	Y	N	N	N	N	N
					Rahnella	N	N	N	N	N	Y
					Serratia	Y	Y	Y	Y	Y	Y
					Yersinia	N	N	N	N	N	Y
				Enterobacteriaceae							
					Enterobacter	Y	N	N	N	N	N
					Lelliottia	N	N	N	N	Y	N
			Pseudomonadales								
				Pseudomonadaceae							
					Pseudomonas	Y	Y	Y	Y	Y	Y
				Moraxellaceae							
					Acinetobacter	Y	N	N	N	Y	N
			Xanthomonadales								
				Rhodanobacteraceae							
					Luteibacter	N	N	Y	N	N	Y
				Xanthomonadaceae							
					Stenotrophomonas	Y	N	N	N	N	Y
					Xanthomonas	N	Y	Y	Y	Y	Y

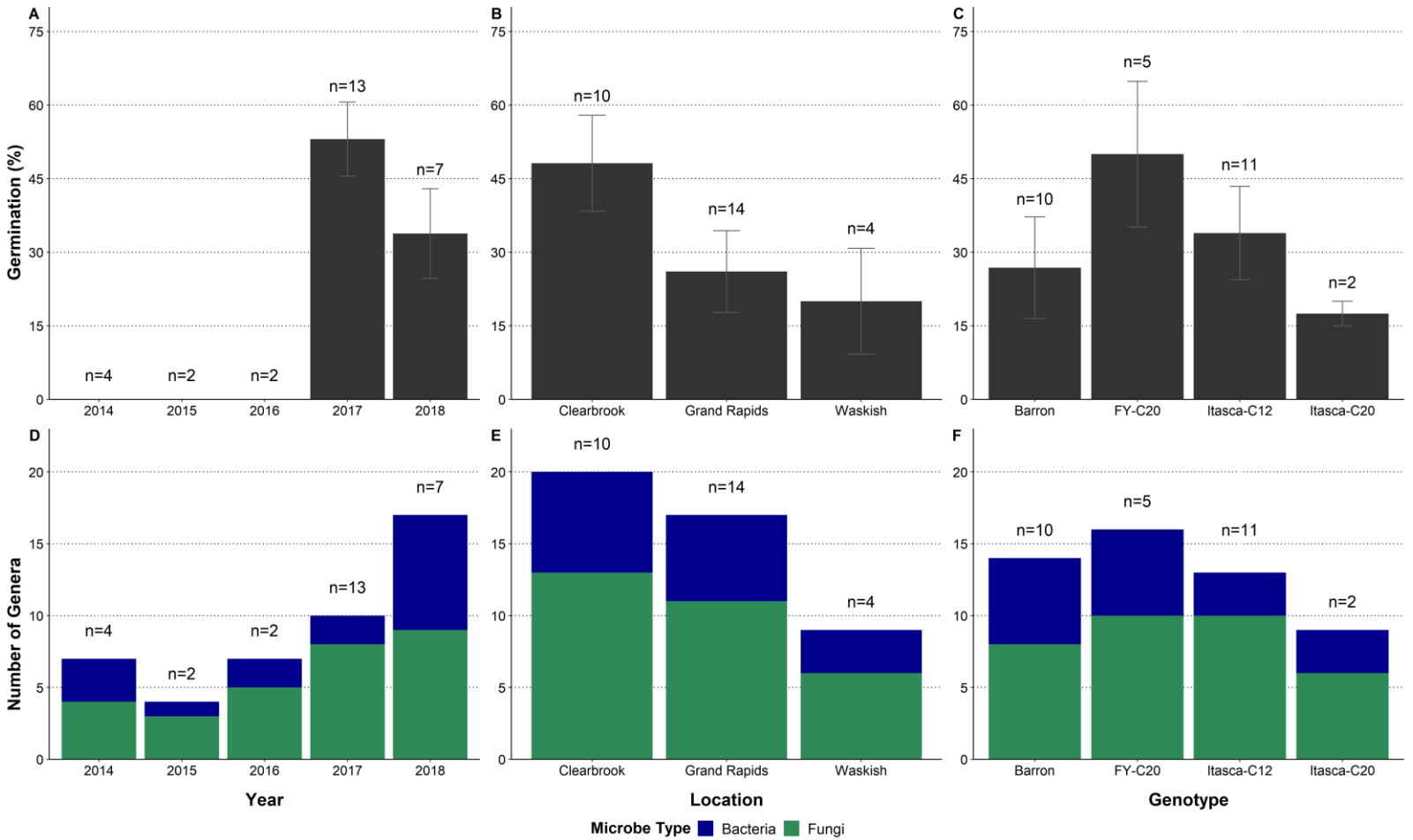

Figure 6 Germination Rates and Microbial Genera. The germination rates as well as microbial genera identified on northern wild rice (NWR; *Z. palustris*) seed stored submerged in water at 3 °C for 1-4 years. A breakdown of germination rates and microbial genera by year (A and D), location (B and E), and by genotype (C and F). The number of seed samples analyzed is denoted above each bar by "n=#."

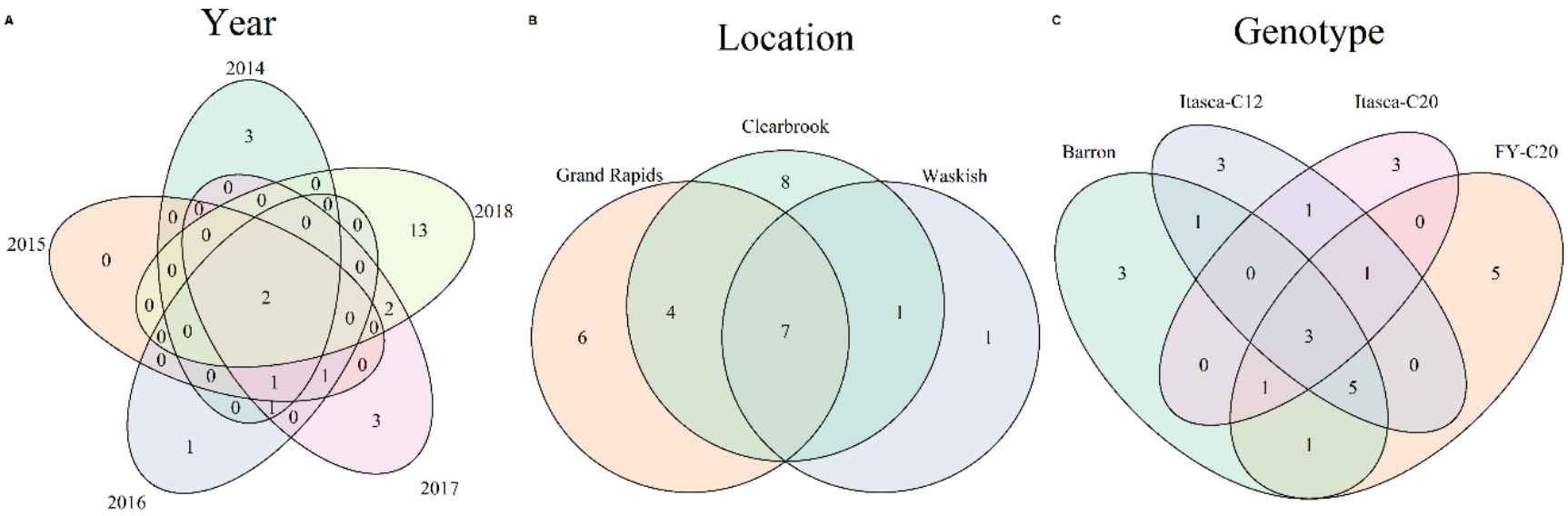

Figure 7 Shared Microbe Venn Diagram. Venn diagrams depicting shared and unique microbes identified on northern wild rice (NWR; *Z. palustris*) seed stored submerged in water at 3 °C for 1-4 years by A) year, B) location, and C) genotype.

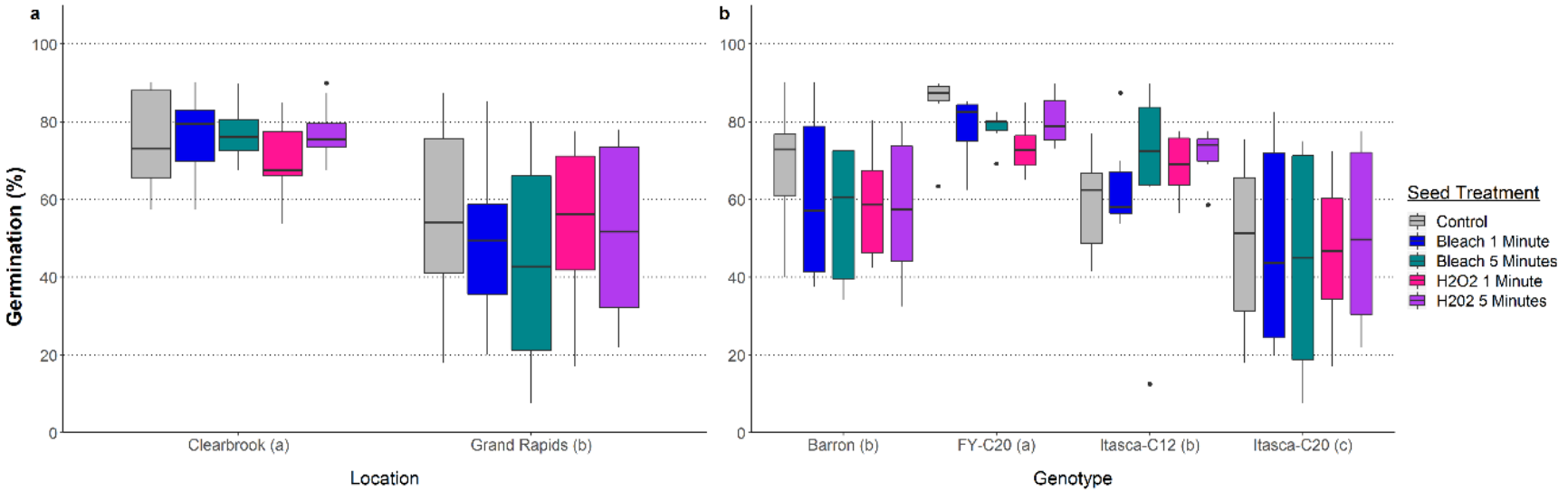

Figure 8 Treated Seed Germination. The germination rates of four northern wild rice (NWR; *Z. palustris*) genotypes from two locations after ~8 months in hydrated storage at 3 °C and post-application of 5 different antimicrobial seed treatments.

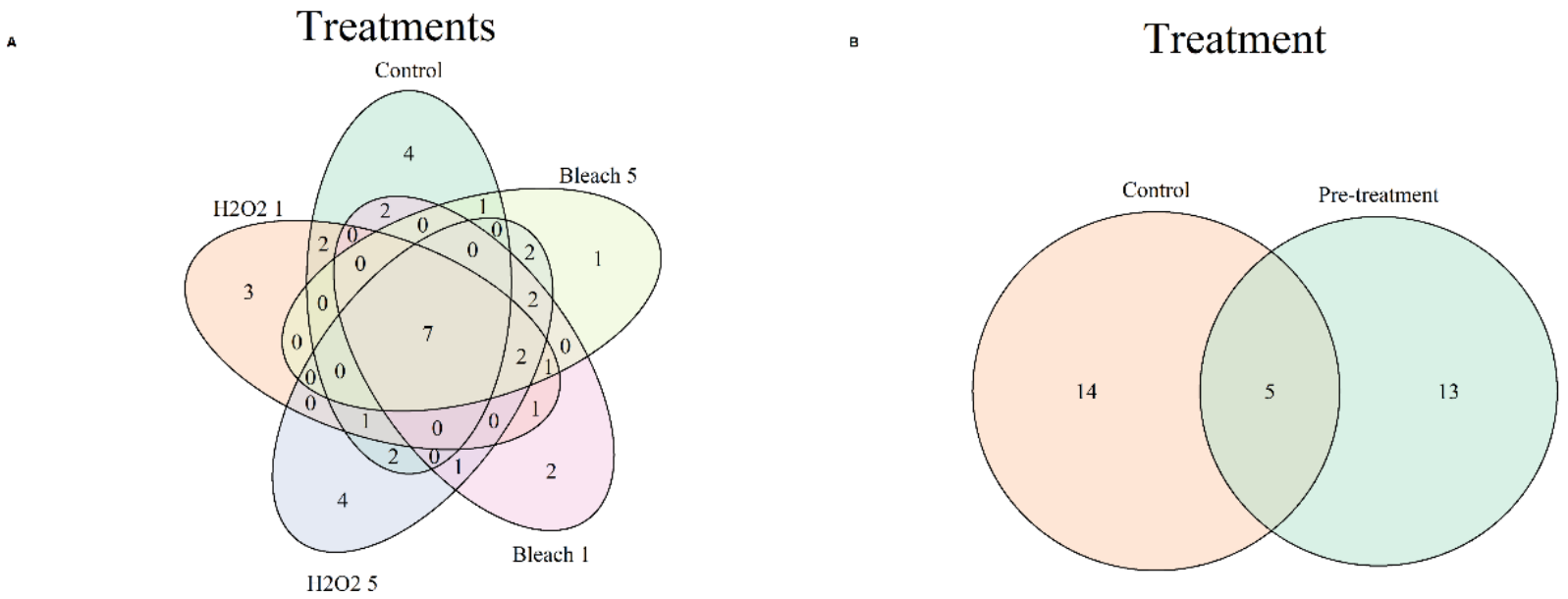

Figure 9 Treatment Shared Microbe Venn Diagram. Venn diagrams depicting shared and unique microbes identified on northern wild rice (NWR; *Z. palustris*) seed stored submerged in water at 3°C for ~ 8 months post-application of 5 different antimicrobial seed treatments by A) control and treatments and B) pre-treatment vs. control.

Table 8 Microbial Genera List by Year, Location, and Genotype. Supplementary tables listing microbes identified on northern wild rice (NWR; *Z. palustris*) seed stored submerged in water at 3 °C for 1-4 years separated by year, location, and genotype. Genera were identified via ITS DNA and 16s rRNA sequencing.

Year	2014	2015	2016	2017	2018
Genera	Bacillus	Geotrichum	Cosmospora	Candida	Achromobacter
	Cosmospora	Penicillium	Flavobacterium	Chryseobacterium	Acinetobacter
	Geotrichum	Pichia	Geotrichum	Cosmospora	Alternaria
	Lysinibacillus	Pseudomonas	Penicillium	Galactomyces	Arthrinium
	Penicillium		Pichia	Geotrichum	Chryseobacterium
	Pseudomonas		Pseudomonas	Mucor	Cladosporium
	Talaromyces			Penicillium	Delftia
				Pichia	Enterobacter
				Pseudeurotium	Epicoccum
				Pseudomonas	Ewingella
					Fusarium
					Mucor
					Penicillium
					Pseudomonas
					Serratia
					Sphingomonas
					Stenotrophomonas

Location	Clearbrook	Grand Rapids	Waskish
Genera	Acinetobacter	Achromobacter	Bacillus
	Alternaria	Bacillus	Candida
	Arthrinium	Candida	Galactomyces
	Bacillus	Chryseobacterium	Geotrichum
	Candida	Cladosporium	Lysinibacillus
	Chryseobacterium	Cosmospora	Mucor
	Cladosporium	Epicoccum	Penicillium
	Cosmospora	Flavobacterium	Pichia
	Delftia	Fusarium	Pseudomonas
	Enterobacter	Geotrichum	
	Ewingella	Mucor	
	Fusarium	Penicillium	
	Galactomyces	Pichia	
	Geotrichum	Pseudeurotium	
	Mucor	Pseudomonas	
	Penicillium	Sphingomonas	
	Pichia	Talaromyces	
	Pseudomonas		
	Serratia		
	Stenotrophomonas		

Genotype	Barron	Itasca-C12	FY-C20	Itasca-C20
Genera	Acinetobacter	Acinetobacter	Achromobacter	Chryseobacterium
	Bacillus	Candida	Acinetobacter	Cladosporium
	Candida	Cladosporium	Alternaria	Delftia
	Chryseobacterium	Cosmospora	Arthrinium	Fusarium
	Cosmospora	Epicoccum	Candida	Mucor
	Enterobacter	Flavobacterium	Chryseobacterium	Penicillium
	Geotrichum	Fusarium	Cosmospora	Pseudomonas
	Lysinibacillus	Galactomyces	Ewingella	Serratia
	Mucor	Geotrichum	Fusarium	Stenotrophomonas
	Penicillium	Mucor	Geotrichum	
	Pichia	Penicillium	Mucor	
	Pseudeurotium	Pichia	Penicillium	
	Pseudomonas	Pseudomonas	Pichia	
	Talaromyces	Talaromyces	Pseudeurotium	
			Pseudomonas	
			Sphingomonas	

Table 9 Seed Inventory List. Supplementary table listing germination rates of all northern wild rice (NWR; *Z. palustris*) genotypes utilized in this study. Results of tetrazolium testing are indicated in the "Metabolically Active" column; seed marked "No" did not stain while seed marked "Yes" did stain.

ID	Year	Location	Genotype	Germination (%)	Metabolically Active
201402	2014	Clearbrook	Barron	0	No
201403	2014	Waskish	Barron	0	No
201432	2014	Grand Rapids	Itasca-C12	0	No
201433	2014	Grand Rapids	Barron	0	Yes
201519	2015	Grand Rapids	Itasca-C12	0	No
201520	2015	Clearbrook	Barron	0	No
201626	2016	Grand Rapids	Barron	0	No
201627	2016	Grand Rapids	Itasca-C12	0	No
201701	2017	Grand Rapids	Barron	40	Yes
201702	2017	Grand Rapids	Itasca-C12	35	Yes
201704	2017	Clearbrook	Itasca-C12	70	Yes
201705	2017	Clearbrook	Itasca-C12	65	Yes
201706	2017	Clearbrook	Itasca-C12	75	Yes
201707	2017	Grand Rapids	FY-C20	80	Yes
201709	2017	Waskish	Itasca-C12	10	Yes
201710	2017	Waskish	Itasca-C12	50	Yes
201711	2017	Waskish	Barron	20	Yes
201712	2017	Clearbrook	Barron	80	Yes
201713	2017	Grand Rapids	FY-C20	10	Yes
201718	2017	Grand Rapids	Barron	75	Yes
201744	2017	Grand Rapids	FY-C20	80	Yes
201801	2018	Grand Rapids	Itasca-C12	5	Yes
201818	2018	Grand Rapids	FY-C20	20	Yes
201827	2018	Grand Rapids	Itasca-C20	20	Yes
201859	2018	Clearbrook	Barron	53.33	Yes
201860	2018	Clearbrook	Itasca-C20	15	Yes
201861	2018	Clearbrook	Itasca-C12	63.3	Yes
201862	2018	Clearbrook	FY-C20	60	Yes

Table 10 R Packages Used. List of R Studio packages used for analysis and figure creation in Chapter 3.

Package	Documentation
VennDiagram	https://cran.r-project.org/web/packages/VennDiagram/VennDiagram.pdf
png	https://cran.r-project.org/web/packages/png/png.pdf
ggpubr	https://cran.r-project.org/web/packages/ggpubr/ggpubr.pdf
ggplot2	https://cran.r-project.org/web/packages/ggplot2/ggplot2.pdf
plyr	https://cran.r-project.org/web/packages/plyr/plyr.pdf
dplyr	https://cran.r-project.org/web/packages/dplyr/dplyr.pdf

Table 11 Microbial Genera List by Treatment. Supplementary tables listing microbes identified on northern wild rice (NWR; *Z. palustris*) seed stored submerged in water at 3 °C post-antimicrobial treatments. Genera were identified via ITS and 16sRNA sequencing.

Treatments	Pre-Treatment	Control	Bleach 1 Min	Bleach 5 Min	Hydrogen Peroxide 1 Min	Hydrogen peroxide 5 Min
Genera	Achromobacter	Chryseobacterium	Alternaria	Alternaria	Acinetobacter	Alternaria
	Acinetobacter	Comamonas	Cadophora	Candida	Chryseobacterium	Candida
	Alternaria	Coniochaeta	Candida	Chryseobacterium	Comamonas	Chryseobacterium
	Arthrinium	Cystofilobasidium	Chryseobacterium	Flavobacterium	Flavobacterium	Cosmospora
	Chryseobacterium	Fusarium	Cladosporium	Fusarium	Fusarium	Fusarium
	Cladosporium	Kluyveromyces	Coniochaeta	Janthinobacterium	Kluyveromyces	Janthinobacterium
	Delftia	Metschnikowia	Flavobacterium	Mucor	Lelliottia	Luteibacter
	Enterobacter	Mucilaginibacter	Fusarium	Novosphingobium	Mucilaginibacter	Metschnikowia
	Epicoccum	Nakazawaea	Luteibacter	Occultifur	Novosphingobium	Mucilaginibacter
	Ewingella	Papiliotrema	Novosphingobium	Papiliotrema	Pedobacter	Mucor
	Fusarium	Phaeotremella	Pedobacter	Pseudomonas	Pseudomonas	Novosphingobium
	Janthinobacterium	Pichia	Pseudomonas	Rhodotorula	Rhodococcus	Pseudomonas
	Mucor	Pseudomonas	Rhodotorula	Serratia	Rhodotorula	Rahnella
	Penicillium	Rhodotorula	Serratia	Sphingobacterium	Serratia	Rhodotorula
	Pseudomonas	Sarocladium	Sphingobacterium	Sphingomonas	Sphingobacterium	Sarocladium
	Serratia	Serratia	Sphingomonas	Xanthomonas	Sphingomonas	Serratia
	Sphingomonas	Sphingomonas	Xanthomonas		Xanthomonas	Sphingobacterium
	Stenotrophomonas	Xanthomonas				Sphingomonas
						Stenotrophomonas
						Xanthomonas
						Yersinia

Location	Clearbrook				
Treatments	**Control**	**Bleach 1 Min**	**Bleach 5 Min**	**Hydrogen Peroxide 1 Min**	**Hydrogen peroxide 5 Min**
Genera	Chryseobacterium	Alternaria	Alternaria	Acinetobacter	Alternaria
	Comamonas	Cadophora	Candida	Chryseobacterium	Candida
	Coniochaeta	Candida	Chryseobacterium	Comamonas	Chryseobacterium
	Kluyveromyces	Chryseobacterium	Flavobacterium	Flavobacterium	Cosmospora
	Metschnikowia	Coniochaeta	Fusarium	Lelliottia	Fusarium
	Nakazawaea	Luteibacter	Janthinobacterium	Novosphingobium	Luteibacter
	Phaeotremella	Pedobacter	Mucor	Pseudomonas	Metschnikowia
	Pichia	Pseudomonas	Occultifur	Rhodococcus	Pseudomonas
	Pseudomonas	Rhodotula	Papiliotrema	Rhodotorula	Rhodotorula
	Rhodotorula	Serratia	Pseudomonas	Serratia	Sarocladium
	Sarocladium	Sphingobacterium	Rhodotorula	Sphingomonas	Serratia
	Serratia	Sphingomonas	Serratia	Xanthomonas	Sphingobacterium
	Sphingomonas	Xanthomonas	Sphingobacterium		Sphingomonas
	Xanthomonas		Sphingomonas		Xanthomonas
			Xanthomonas		

Location	**Grand Rapids**				
Treatments	**Control**	**Bleach 1 Min**	**Bleach 5 Min**	**Hydrogen Peroxide 1 Min**	**Hydrogen peroxide 5 Min**
Genera	Coniochaeta	Chryseobacterium	Chryseobacterium	Fusarium	Chryseobacterium
	Cystofilobasidium	Cladosporium	Flavobacterium	Kluyveromyces	Fusarium
	Fusarium	Flavobacterium	Mucor	Mucilaginibacter	Janthinobacterium
	Kluyveromyces	Fusarium	Novosphingobium	Novosphingobium	Metschnikowia
	Mucilaginibacter	Novosphingobium	Pseudomonas	Pedobacter	Mucilaginibacter
	Papiliotrema	Pseudomonas	Rhodotorula	Pseudomonas	Mucor
	Pseudomonas	Rhodotorula	Sphingomonas	Sphingobacterium	Novosphingobium
	Rhodotorula	Sphingobacterium		Sphingomonas	Pseudomonas
	Sphingomonas	Sphingomonas		Xanthomonas	Rahnella
					Sphingobacterium
					Sphingomonas
					Stenotrophomonas
					Yersinia

Genotypes	**Barron**				
Treatments	**Control**	**Bleach 1 Min**	**Bleach 5 Min**	**Hydrogen Peroxide 1 Min**	**Hydrogen peroxide 5 Min**
Genera	Chryseobacterium	Chryseobacterium	Chryseobacterium	Fusarium	Chryseobacterium
	Kluyveromyces	Comamonas	Fusarium	Luteibacter	Mucor
	Nakazawaea	Pedobacter	Janthinobacterium	Pseudomonas	Novosphingobium
	Pseudomonas	Pseudomonas	Luteibacter	Rhodotorula	Pseudomonas
	Sphingomonas	Sphingomonas	Metschnikowia	Sphingobacterium	Rhodotorula
	Xanthomonas	Xanthomonas	Pseudomonas	Sphingomonas	Xanthomonas
			Serratia		
			Sphingomonas		

Genotypes	**FY-C20**				
Treatments	**Control**	**Bleach 1 Min**	**Bleach 5 Min**	**Hydrogen Peroxide 1 Min**	**Hydrogen peroxide 5 Min**
Genera	Chryseobacterium	Lelliottia	Alternaria	Alternaria	Candida
	Coniochaeta	Mucilaginibacter	Candida	Cadophora	Fusarium
	Cystofilobasidium	Pseudomonas	Chryseobacterium	Candida	Janthinobacterium
	Metschnikowia	Rhodotorula	Cosmpospora	Chryseobacterium	Papilotrema
	Phaeotremella	Serratia	Fusarium	Coniochaeta	Pseudomonas
	Pichia	Sphingobacterium	Pseudomonas	Pseudomonas	Sphingomonas
	Pseudomonas	Sphingomonas	Rhodotorula	Rhodotula	
	Rhodotorula	Xanthomonas	Sphingobacterium	Sphingomonas	
	Serratia		Sphingomonas		
	Sphingomonas				

Genotypes	Itasca-C12				
Treatments	**Control**	**Bleach 1 Min**	**Bleach 5 Min**	**Hydrogen Peroxide 1 Min**	**Hydrogen peroxide 5 Min**
Genera	Comamonas	Comamonas	Metschnikowia	Chryseobacterium	Alternaria
	Fusarium	Fusarium	Mucilaginibacter	Fusarium	Chryseobacterium
	Pseudomonas	Kluyveromyces	Novosphingobium	Luteibacter	Flavobacterium
	Sphingomonas	Novosphingobium	Pseudomonas	Novosphingobium	Occultifur
		Pseudomonas	Sarocladium	Pedobacter	Papiliotrema
		Serratia	Serratia	Pseudomonas	Pseudomonas
		Sphingomonas	Sphingomonas	Sphingomonas	Serratia
			Stenostrophomonas	Xanthomonas	Sphingomonas
			Xanthomonas		Xanthomonas
					Yersinia

Genotypes	Itasca-C20				
Treatments	**Control**	**Bleach 1 Min**	**Bleach 5 Min**	**Hydrogen Peroxide 1 Min**	**Hydrogen peroxide 5 Min**
Genera	Coniochaeta	Acinetobacter	Fusarium	Cladosporium	Chryseobacterium
	Fusarium	Novosphingobium	Mucilaginibacter	Flavobacterium	Mucor
	Mucilaginibacter	Pseudomonas	Mucor	Pseudomonas	Pseudomonas
	Papiliotrema	Rhodococcus	Pseudomonas	Serratia	Rhodotorula
	Pichia	Rhodotorula	Rahnella	Sphingobacterium	Sphingobacterium
	Pseudomonas	Sphingobacterium	Serratia	Sphingomonas	Sphingomonas
	Serratia	Sphingomonas	Sphingobacterium		Xanthomonas
	Sphingomonas		Sphingomonas		
			Stenotrophomonas		

Table 12 ITS and 16sRNA Primer Sequences. List of ITS DNA and 16s rRNA primer sequences used in this study.

Primer name	Primer sequence (5′-3′)
ITS1F	CTTGGTCATTTAGAGGAAGTAA
ITS4	TCCTCCGCTTATTGATATGC
27F	AGAGTTTGATCMTGGCTCAG
1492R	RGYTACCTTGTTACGACTT

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Appendix 1 - Testing Commercial Antimicrobial Seed Treatments in Northern Wild Rice (*Z. palustris* L.)

Objectives

The objectives of this study were to expand on the 2018 antimicrobial treatment findings, to identify an antimicrobial treatment rinse that reduced microbial growth in storage and maintained high seed viability. Additionally, commercial fungicidal seed treatments were explored to understand their impact on microbial growth in intermediate/recalcitrant, aquatic seed.

Materials and Methods

Treatments. Antimicrobial seed treatments were expanded in 2019. After seeing widespread microbial growth after treatment in 2018, rinse times were lengthened from 1 and 5 minutes in 2018 to 5 and 10 minutes in 2019. As in 2018, all treatments (with the exception of an untreated control) received a 1 minute ethanol rinse. A 10% bleach solution and tween 20 was used again as well. Treatments consisted of 10% bleach/tween 20 rinses for 5 or 10 minutes, 0.1% mercuric chloride rinses for 5 or 10 minutes, and two fungicide products that remained in the storage container for the duration of the dormancy period.

Seed Rinse Protocol. Three replications of 100 seeds per genotype were counted out by mass and placed into 50 ml falcon tubes. Seeds were covered with 70% ethanol and rinsed/agitated for one minute to aid in the breakdown of the seed's waxy coating. Ethanol was poured off and then seeds were covered with the appropriate treatment solution and agitated for the designated rinse time (5 or 10 minutes). In the case of the bleach, mercuric chloride, and control treatments, treatment solution was poured off, and seeds were rinsed three times with deionized water. Tubes were filled to the 35 ml line with DI water one final time and placed in dark storage at 3°C for 27 weeks until germination testing.

Fungicidal Seed Treatment (FST) Protocol. FSTs were applied at the labeled rate. For the first FST, a premix formulation of prothioconazole at 0.075 g/kg seed, tebuconazole at 0.015 g/kg seed, and metalaxyl at 0.031 g/kg seed (Raxil® PRO MD, Bayer CropScience, St. Louis, MO) was added to 500µl deionized water which was then added to a 50ml falcon tube containing 100 seeds. Seed tubes were agitated to coat seed with the treatment slurry. Prior to storage, deionized water was added to the seed tube to the 35ml line. For the second FST treatment, a premix formulation of trifloxystrobin at 0.100 g/kg seed and metalaxyl at 0.079g/kg seed (Trilex® 2000, Bayer CropScience, St. Louis, MO) was added to 500µl deionized water which was then added to a 50ml falcon tube containing 100 seeds. Seed tubes were agitated to coat seed with the treatment slurry and deionized water was added

to the 35ml line of the falcon tube. Treated tubes were then placed in dark storage at 3°C for 27 weeks until germination testing.

Seed tube observations. Throughout the storage period, treatment tube observation data was collected. Jars were monitored for microbial growth symptoms like water color change and water turbidity, as well microbial growth signs like floating fungal masses.

Germination Testing. Germination testing was analyzed 27 weeks after treatment to test the viability of treated seed. Forty seeds from each treatment tube were placed in a ULINE 2mil 3”x5” plastic sample bag and filled with 10ml deionized water. Bags were placed under a benchtop grow light programmed for 16 hour days. Ambient temperature was approximately 22°C. Seeds were monitored for 14 days. A germinated seedling was defined as a seedling with a greening coleoptile that surpassed 1 cm in length. Water in bags was changed every two days to reduce seedling deterioration due to microbial growth.

Data Analysis. Analysis of variance was generated using R with fixed effects for replication, genotype, and treatment. Using the LSD test function with the Bonferroni adjustment, least significant differences were calculated for genotype and treatment.

Results and Discussion

Germination Data. Differences in germination between genotypes and treatments were found in 2019 (Table 13). Germination ranged from 42.5% to 97.5% across all genotypes and treatments. The seed treatments in this study did appear to have an effect on germination. Only the 10 minute rinse of mercuric chloride resulted in a higher germination rate (90.7%) relative to the untreated control (81.6%; Figure 10). The 10 minute rinse of mercuric chloride, 5 minute rinse of mercuric chloride, 5 minute rinse of bleach, the surface-sterilized control, and the premix formulation of prothioconazole, tebuconazole, and metalaxyl (PTM fungicide) all were among the higher germinating group (Figure 10). Genotype also affected germination (Figure 11). When averaged across treatments, Barron had the highest germination rate at 90.2%, followed by elite breeding line FY-C20 (84.6%) and Itasca-C20 (84.3%). The lowest germinating genotype was Itasca-C12 (76.5%). It should be noted that NWR genotype germination fluctuates from year to year, meaning that genotype germination differences may be decreased or eliminated with multi-year data.

Ultimately, the methodology for treating recalcitrant or intermediate seed that is stored submerged needs to be explored further. In other recalcitrant species, seed was treated and then either not stored submerged, or retreated throughout the storage period all with varying levels of success (Lacerda et al., 2016; Silva et al., 2011). Treatment solution rinses may be effective in research settings, but could be time consuming if implemented program-wide postharvest. Determining the most effective timing of treatment will be

essential to the successful use of treatment solutions in NWR. In commercial settings, additional research must be done on environmental impact of treatment solutions if recommendations are made to growers, as seed is not stored in a closed system as it is in research settings. Mercuric chloride is an effective antimicrobial solution (Abraham, Raju, Suresh, & Damodharam, 2012; Ramakrishna, Lacey, & Smith, 1991), but is toxic to humans and wildlife (Elblehi, Hafez, & El-Sayed, 2019) and thus may not be a realistic treatment solution. However, it represented a positive check in the sense that it showed it was possible to see significant improvements in germination rate from untreated controls.

This experiment showed that FST use in seed stored submerged in water is likely either ineffective or was not implemented correctly. While FSTs are used commonly in other crops (Lamichhane et al., 2020; White & Hoppin, 2004), FST use has seen little exploration in recalcitrant or intermediate seed. The two FSTs studied are labeled for control of microbial genera that were identified in 2018. Submerged storage conditions continue to make NWR germplasm storage difficult. Ultimately, developing a protocol to improve storage conditions should take higher precedence in the breeding program, as research in other recalcitrant/intermediate species suggests seed treatments are more effective and easier to implement when seed is not stored submerged.

Table 13 Seed Treatment Germination ANOVA. Analysis of Variance between eight treatments, four genotypes, and their interaction on 2019 northern wild rice seed.

Source of Variation	Df	Sum Sq.	Mean Sq.	F value	Pr (>F)
Replication	2	0.00146	0.00073	0.166	0.84707
Treatment	7	0.11555	0.01651	3.754	0.00188**
Genotype	3	0.229984	0.7661	17.424	2.57e-08***
Treatment x Genotype	21	0.14801	0.00705	1.603	0.07794
Residuals	62	0.27262	0.0040		

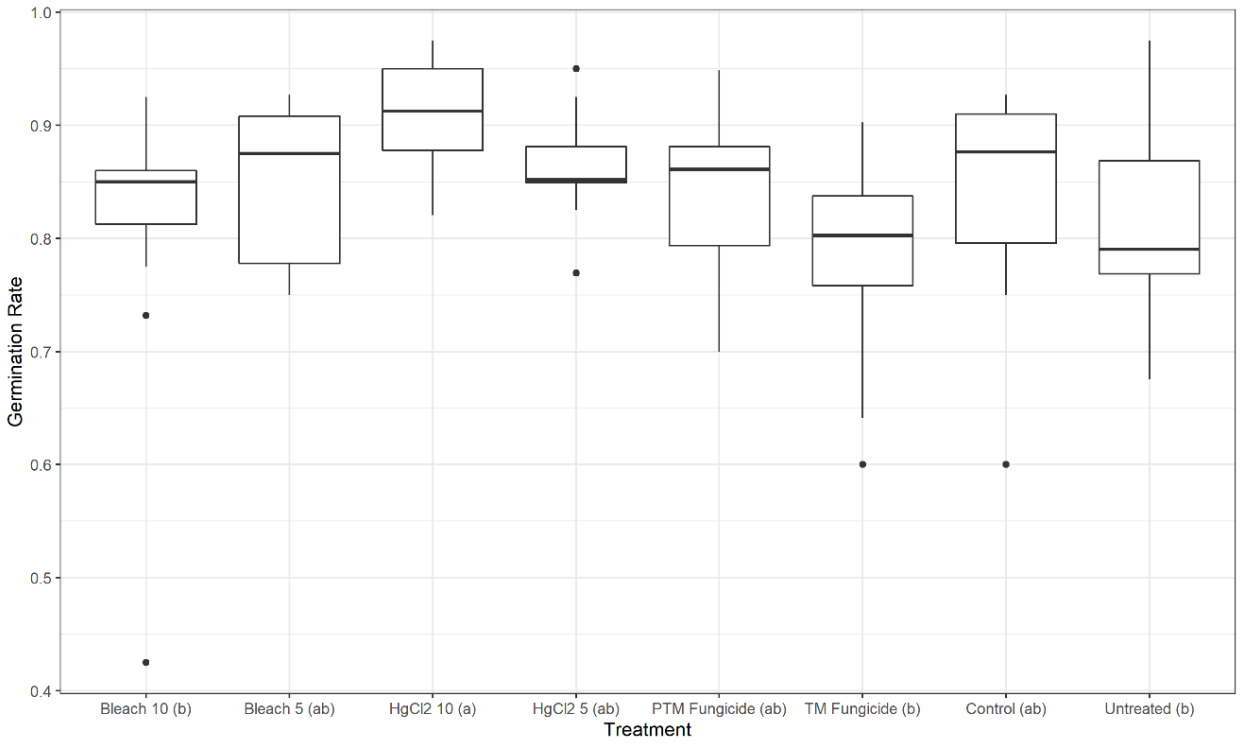

Figure 10 Average Germination Rate Across Treatments in 2019. Box plots showing germination rates across eight seed treatments. Treatments included 10% bleach for 5 or 10 minutes rinses, 0.1% mercuric chloride for 5 or 10 minute rinses, a fungicide seed treatment containing prothioconazole, tebuconazole, and metalaxyl, a fungicide seed treatment containing trifloxystrobin and metalaxyl, an ethanol treated/surface sterilized control, and a completely untreated control. The bold line within each box represents the median germination for that treatment.

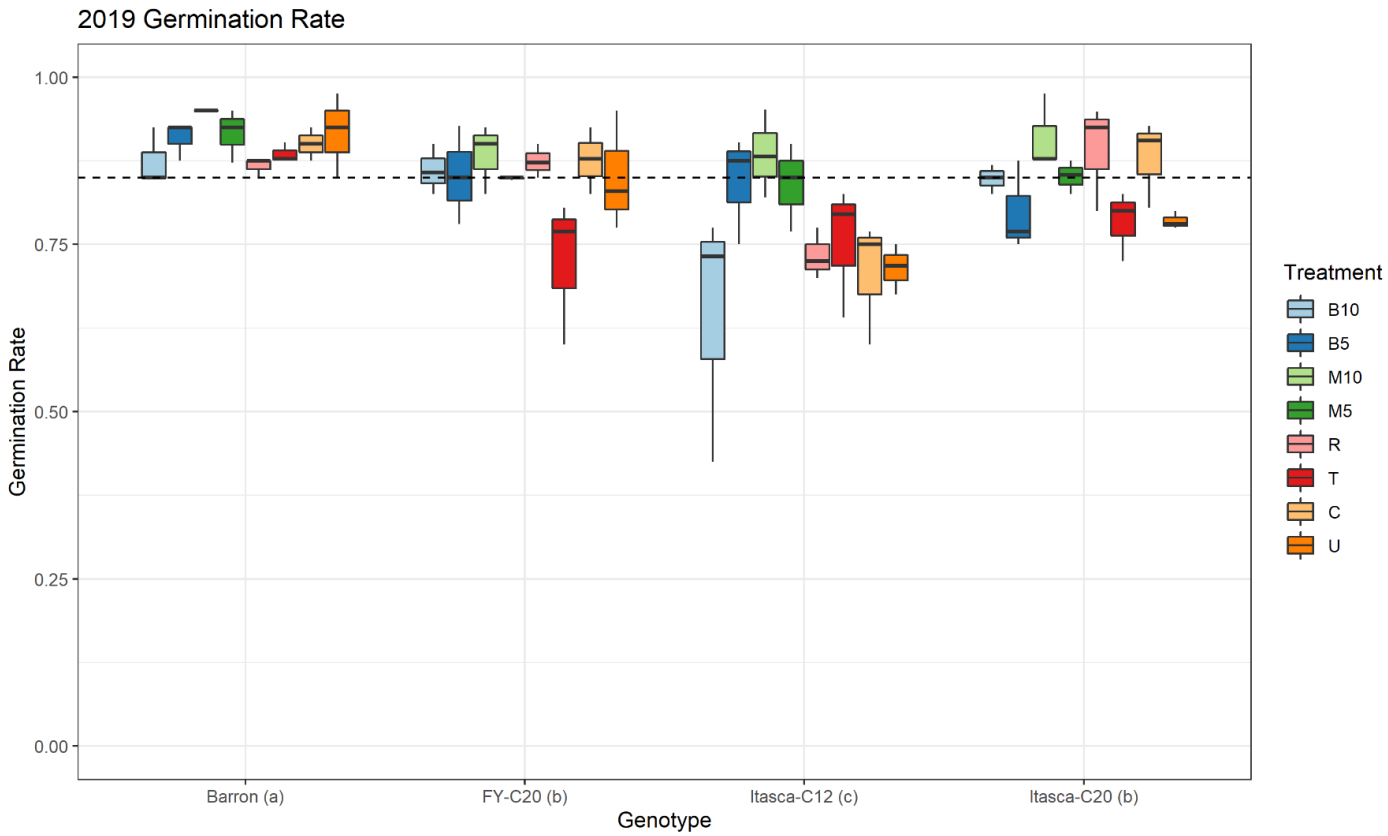

Figure 11 Germination Rate of Genotype x Treatment from 2019. Germination rate of four northern wild rice genotypes separated by treatment. Average germination is signified by the dashed line. Treatments include included 10% bleach for 5 or 10 minutes rinses, 0.1% mercuric chloride for 5 or 10 minute rinses, a fungicide seed treatment containing prothioconazole, tebuconazole, and metalaxyl, a fungicide seed treatment containing trifloxystrobin and metalaxyl, an ethanol treated/surface sterilized control, and a completely untreated control.